REMARKS

The above-identified application is a divisional application of U.S. Patent Application Serial No. 09/363,678, filed on July 30, 1999.

Status of claims

Claims 1-14, 21, and 22 were originally filed in the application. In response to the Restriction Requirement mailed on May 7, 2003, Applicant elected to prosecute the claims of Group II (claims 21 and 22), drawn to PBMC and a population of T-cells. Claims 21 and 22 are currently pending in the application.

Rejection of claims 21 and 22 under 35 U.S.C. §§ 102(e) and 102(b)

Claims 21 and 22 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Bolton (U.S. Patent No. 5,980,954; henceforth, "the '954 patent"). In particular, the Office Action asserts that "the '954 patent teaches a population of mammalian T-cells (peripheral blood mononuclear cells) essentially free of stem cells..." Office Action at page 2, paragraph 4.

Claims 21 and 22 were also rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by WO 98/07436, for the same reasons. The Office Action refers to page 8, second paragraph, of the published PCT application. Office Action at page 2, paragraph 5.

Applicant traverses both rejections, which will be addressed together.

Applicant first notes that the term "peripheral blood mononuclear cells" (PBMC) refers to a population of cells that mainly comprise circulating T and B-lymphocytes. PBMC are not synonymous with or coextensive in scope with "mammalian T-cells," as the Office Action would appear to imply by the language at paragraphs 4 and 5. Applicant submits, herewith, portions of reference texts relating to cell culture methods for the study of animal cells or immune cells. Each of these references describes one or more methods for preparing PMBC using Ficoll-Hypaque or Percoll density gradients, which appears to be the most common method of preparing PBMC. Each reference explicitly states that the resulting mononuclear cell

populations comprise lymphocytes as well as other mononuclear cells.[1] Note that additional steps are necessary to fractionate B and T-cells from PBMC.[2] Thus, "PBMC" is not synonymous with "mammalian T-cells." The apparent failure of the Office Action to recognize the distinction between these claim terms is troubling since the rejections under 35 U.S.C. § 102 appear to be based on the particular constituent cells present in the cell populations described in the cited prior art and in the instant application.

This distinction aside, Applicant submits that the cited prior art does not disclose the use of either PBMC or mammalian T-cells, much less such cell populations that are "essentially free of stem cells." Specifically, neither the '954 patent, at column 4, line 65 – column 5, line 4, nor the WO 98/07436 application, at page 8, paragraph 2, recite the use of PBMC or mammalian T-cells, as alleged in the Office Action. In fact, the cited text of the two Specifications refers to "an aliquot of blood of volume about 0.01 ml to about 400 ml." Treatment of an aliquot of blood is also recited in the '954 patent, *e.g.*, at column 4, lines 15-35 and the WO 98/07436 application, *e.g.*, at page 8, lines 9, 11, 13, 30, 32; page 6, lines 15-17; page 10, lines 18-25; and page 11, lines 1-5. While the Specification of the '954 patent refers generally to the use of "separated cellular fractions of the blood" (column 8, lines 57-61), there is no specific mention of PBMC or fractions of blood that are "essentially free of stem-cells."

Moreover, the terms "PBMC," "peripheral blood mononuclear cells," and/or "stem cells" are completely absent in the '954 patent and WO 98/07436 application. There is therefore no factual basis for the rejection of claims 21 and 22 under 35 U.S.C. §§ 102(e) and 102(b), as set forth at paragraphs 4 and 5 of the Office Action. For at least these reasons, Applicants submit that the rejection is improper and should be withdrawn.

Additionally, there is abundant evidence in the Specifications of the cited references to convince one skilled in the art that the cell populations described for use in preferred embodiments of the respective inventions are not PBMC or mammalian T-cells (the presence or

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[1] See e.g., Exhibit 1 (de Waele, M. and Beesley, J.E., "Immunochemistry of blood and bone marrow cells," *in Techniques in Immunochemistry*, Vol. 4., Bullock, G.R. and Petrusz, P., *ed.*, Academic Press, 1989) at page 98, lines 25-35; Exhibit 2 (Ali, F.M.K., *Separation of Human Blood and Bone Marrow Cells*. IOP Pub, Ltd., 1986) at page 62, lines 5-7; page 63, lines 13-17; page 64, lines 1-7; page 66, lines 3-12; and the remainder of Chapter 3, which describes methods of further separating component cell population present in PBMC preparations; and Exhibit 3 (Freshney, R.I. *Culture of Animal Cells: A Manual of Basic Techniques*. Alan R. Liss, Inc., 1987) at page 318, last paragraph (note that only the relevant pages of the Chapter entitled "Specialized Techniques" were included.

[2] See e.g., Exhibit 2 (Ali at page 77.).

absence of stem cells aside). In particular, the presence of neutrophils in the cells treated by the invention is also indicated at column 6, line 59; and page 12, line 19, of the '954 patent and WO 98/07436 application, respectively. Neither PBMC nor mammalian T-cells would be expected to neutrophils. It is therefore clear that the cell populations described in the cited prior art are not PBMC or mammalian T-cells.

In view of the evidence presented above, it is clear that the prior art references cited in the Office Action do not teach the use of "mammalian T-cells (peripheral blood mononuclear cells) essentially free of stem cells." First, the '954 patent and WO 98/07436 application specifically describe the presence of neutrophils in the aliquot of blood to be treated according to the respective inventions. Additionally, neither of these Specifications recite the terms "stem cells," "peripheral blood mononuclear cells," or "PBMC." Applicant therefore submits that the rejections under 35 U.S.C. §§ 102(e) and 102(b) are not supported factually by the evidence and should be withdrawn.

CONCLUSION

For the above stated reasons, Applicant submits that the rejections based on the cited prior art documents should be withdrawn. Applicant further submits that the instant application is now fully in condition for allowance. Early notice to that effect is earnestly solicited. Should a telephone conversation expedite allowance of the application, the Examiner is invited to call the undersigned.

Respectfully submitted,

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PLATE 22. Retrograde labeling combined with GLU 1CC in the rat cerebral (motor) cortex. A complex of wheat germ agglutinin, inactivated horseradish peroxidase, and colloidal gold (Basbaum and Menetrey, 1987) was injected into the spinal cord of a rat. Four days after the injection, the rat was sacrificed by perfusion with 4% carbodiimide followed by 4% paraformaldehyde. Vibratome sections of the motor cortex were processed as described in the text. Four populations of neurons can be distinguished (representative examples are indicated in the figure by the corresponding numbers): (1) double-labeled, i.e., neurons which are both GLU + (brown DAB reaction product) and corticospinal (dark colloidal gold–silver granules); (2) single-labeled with brown DAB reaction product (GLU+ but not corticospinal); (3) single-labeled with dark colloidal gold–silver granules (corticospinal but not GLU +); and (4) unlabeled neurons which stain purple with thionin (neither corticospinal nor GLU +). Scale bar: 20μm. This micrograph was kindly provided by Rosario Giuffrida.

Immunocytochemistry of Blood and Bone Marrow Cells

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I. INTRODUCTION

Blood cells are derived from a common precursor: the pluripotent hemopoietic stem cell (Chervernick and Zucker-Franklin, 1981). This stem cell differentiates into cells which further develop into one of the five major cell types: erythrocytes, megakaryocytes, granulocytes, mononuclear phagocytes and lymphocytes. These cells are found in the hematopoietic tissue at various stages of maturation and development. Normoblasts, megakaryocytes and immature granulocytes are localized in the bone marrow, while the erythrocytes, platelets and polymorphonuclear leukocytes are present in the blood. Promonocytes in the bone marrow give rise to blood monocytes, which eventually migrate into various organs to form the phagocytic macrophages. Antigen presenting cells, like the Langerhans cells in the skin, the interdigitating cells and the follicular dendritic cells in the lymph nodes and the spleen and the interdigitating follicular cells in the thymic medulla, are also bone marrow derived (Roitt *et al.*, 1985). Lymphocyte precursors differentiate into B- and T-cells respectively in the bone marrow and the thymus. These cells migrate through the blood to the secondary lymphoid organs such as the lymph nodes, the spleen and the lymphoid tissue of the alimentary and the respiratory tracts (Roitt *et al.*, 1985). Upon antigenic stimulation these cells undergo morphological changes and differentiate into various types of effector and memory cells. These lymphocytes may re-circulate through blood and lymphatic ducts between various lymphoid organs. A third population of lymphocytes are the non-T, non-B or null cells, which contain the majority of natural killer and antibody-dependent cellular cytotoxic effector cells.

These blood and bone marrow cells are classically identified by their morphological and cytochemical characteristics, their distribution in the hematopoietic tissues and their functional activities. A more recent approach for this purpose is the study of their antigen and receptor phenotype (Sun *et al.*, 1985). Cell surface and intracytoplasmic antigens are detected with specific antibodies and immunocytochemical markers. Antibodies defining the cell lineage, the stage of maturation, the state of activation or the functional characteristics of the cells have been developed (Shaw, 1987; Pallesen and Plesner, 1987). In the diagnostic hematological laboratory these antibodies are used to enumerate leukocyte subpopulations for the evaluation of the immunoregulatory status of an individual (Reinherz and Schlossman, 1981; Bach and Bach, 1981). In addition, they are applied for the identification and classification of leukaemic cells (Foon and Todd, 1986). In this chapter we will review the different immunocytochemical procedures which are used for this purpose.

II. SAMPLES

Peripheral blood is collected in tubes containing 5% (w/v) EDTA or 10–20 U/ml preservative-free heparin. Peripheral blood can also be defibrinated which removes all the platelets. Bone marrow aspirates can be collected in an equal volume of sterile PBS at pH 7·4 or in culture medium (e.g. RPMI) supplemented with 10–20% fetal calf serum. Five per cent EDTA may be used as anticoagulant. These samples are kept at room temperature, for not longer than 24 h before processing. Storage at 4°C or for more than 24 h at room temperature may induce changes in the cell surface phenotype of the cells (Milson and Keller, 1982; Patrick *et al.*, 1984). Complex holding media for these samples have been described which produce only minor changes of the cell surface phenotype for 48 h after collection although morphological changes may occur (Milson *et al.*, 1986).

Biological fluids and lavages are collected without anticoagulant. The cells in these samples are relatively fragile and should be processed as soon as possible.

Biopsies of lymphoid tissues are kept unfixed in PBS at pH 7·4, eventually supplemented with 5% fetal calf serum, at 4°C for a maximum 24 h (Palutke *et al.*, 1986).

III. CELL PREPARATION

A. Cell Suspensions

Leukocyte suspensions can be prepared in various ways from peripheral blood and bone marrow (Jackson and Warner, 1986). With peripheral blood, a total leukocyte count and differential may be performed allowing the calculation of recovery and of the absolute values of the different leukocyte subpopulations.

1. Peripheral Blood

(a) Lysis of the red blood cells

The red blood cells in the samples may be lysed by a hypotonic medium or an ammonium chloride lysis buffer (Hoffman *et al.*, 1980; Parker and Haslam, 1988). Nucleated red blood cells are not lysed by these methods. Ammonium chloride treatment lyses more erythrocytes than the hypotonic medium and produces debris of smaller size. An increase in the lysis time with ammonium chloride may result in cell death and alteration of the

morphology of the cells. A high red cell removal was reported with a methanol-based lysis and fixation solution, but changes in the cell morphology were also seen (Parker and Haslam, 1988). These suspensions contain all leukocyte types and a variable amount of red blood cells and debris. The presence of this debris and of erythrocyte ghosts may result in difficulties for the selection of cell "gates" in flow cytometry. Attempts have been made to remove the debris by washing steps with centrifugations or by centrifuging the cells over a layer of bovine serum albumin or newborn calf serum (Jackson and Warner, 1986). The erythrocyte lysis may be performed before or after the immunostaining of the leukocytes. Staining, lysis and analysis of the sample can all be performed in the same tube which minimizes any biohazardous exposure of the samples.

These rapid whole blood-lysis procedures are used mainly for flow cytometry or for samples containing low numbers of cells where other separation methods would result in considerable cell loss.

(b) Buffy coat centrifugation or dextran sedimentation

Leukocyte suspensions can also be prepared by buffy coat centrifugation (1500 × *g*; 15 min) or by dextran sedimentation. In the latter procedure 1 ml of blood is mixed with 0·2 ml of 5% dextran solution (MW 200 000) in isotonic saline for 30 min at 37°C. The red cells sediment and the leukocyte-rich supernatant is then collected and washed. These suspensions usually contain all leukocyte types and a variable amount of red blood cells. These preparation methods are relatively simple and are often used for the preparation of cell smears for immunostaining.

(c) Ficoll-Hypaque centrifugation

A mononuclear cell suspension can be prepared by centrifugation of peripheral blood diluted 1:2 with PBS over Ficoll-Hypaque (Boyum, 1968) at 400 × *g* for 40 min at 20°C. Erythrocytes, granulocytes and dead cells will be found at the bottom of the tube. At the interface between the plasma and the Ficoll-Hypaque, a layer of cells containing approximately 80% lymphocytes and 20% monocytes will be found. After collecting these cells, contaminating platelets can be removed by a washing step with low speed centrifugation (100 × *g*; 10 min) and removal of the platelet-rich supernatant. A recovery of 70–80% of the leukocytes is possible with this method.

Commercial separator tubes (Leuco-Prep, Becton Dickinson, Sunnyvale, CA, USA) are now available which contain a semi-rigid gel and Ficoll. Undiluted blood is brought on the top of the gel and only a short centrifugation (10 min at 900 × *g*) is needed for cell separation (Gadol *et al.*, 1985).

2. Bone Marrow

In a bone marrow aspirate, the floating particles are collected in buffer and the cells are shaken out on a Vortex. A buffy coat or a mononuclear cell suspension can then be prepared from this suspension. The cells in the particles represent true bone marrow cells. The rest of the bone marrow aspirate can also be used, especially when only very few particles are present, but they may contain many contaminating peripheral blood cells.

3. Biopsies of Lymphoid Tissue

A cell suspension can be made from fresh unfixed biopsies of lymphoid tissue. This is usually done by cutting the tissue in small pieces and shaking the cells out in PBS or culture medium on a Vortex or by pressing the pieces against a mesh (Ford and Hunt, 1978). If the red cell contamination is high, such as with a spleen biopsy, an additional lysis of the red blood cells or a centrifugation over Ficoll-Hypaque may be performed.

4. Preparation of the Cell Suspension for Immunostaining

All cell suspensions are washed with PBS at pH 7·4 by repeated centrifugation and resuspension. Standardized cell suspensions of maximum 30×10^6 cells/ml are then prepared in PBS often supplemented with 5% bovine serum albumin (BSA).

The viability of the cells is determined with a trypan blue exclusion test (Ford and Hunt, 1978) or by staining the cells with ethidium bromide or propidium iodide at a concentration of 1 μg/ml for 1 min (Johnson and Holborow, 1986). The viability should be at least 80% before immunostaining since non-viable cells may stain non-specifically.

Cell suspensions may be stored by freezing in culture medium (e.g. RPMI) supplemented with serum or in the presence of dimethylsulfoxide. The cells are frozen in liquid nitrogen or in a −70°C freezer. Caldwell *et al.* (1987) did not find significant differences in the relative proportions of the lymphocyte subsets after 90 days of frozen storage. In contrast Prince and Lee (1986) noted significant changes after only 4 weeks of preservation.

B. C ll Smears and Touch Imprints

Smears can be prepared from peripheral blood and bone marrow aspirates. Likewise smears or cytocentrifuge preparations can be made from leukocyte suspensions. A good cell density and a good morphology is obtained in cytocentrifuge preparations when 100 µl of a cell suspension containing $0{\cdot}5 \times 10^6$ to $1{\cdot}5 \times 10^6$ cells per ml are centrifuged (Shandon cytocentrifuge, Shandon Southern Instruments, Sewickley, Pennsylvania) at 500–800 rpm for 5–7 min (Yam *et al.*, 1987). A small quantity of protein, e.g. 5% BSA, can be added to the cell suspension to improve the cell adhesion to the microscope slide. For the staining of immunoglobulin on B-lymphocytes the suspension should be washed at least once to remove all plasma immunoglobulins before making the cytocentrifuge preparations.

Imprints can be made from an unfixed biopsy of lymphoid tissue by gently touching a microscope slide with the fresh cutting side of the tissue.

All these preparations are air dried for between 2 and 24 h by leaving them exposed to the air at ambient temperature. They can then be labeled immediately or stored at −20°C, wrapped in parafilm or aluminum foil (Moir *et al.*, 1983).

IV. SPECIFIC ANTIBODIES

A. M n cl nal Antibodies

A whole panel of monoclonal mouse antibodies directed against leukocyte cell surface antigens has been described (Shaw, 1987; Pallesen and Plesner, 1987). These antibodies identify the cell lineage, the stage of maturation, the state of activation or the functional capacities of the cells. Based on their reactivity with selected leukocyte suspensions and on the molecular weight of the antigen, these antibodies have been classified in clusters of differentiation (CD groups) by Workshops on Human Leukocyte Differentiation Antigens. So far, 45 different CD groups have been established. For each of these CD groups several monoclonal antibodies are commercially available. Examples of frequently used monoclonal antibodies are mentioned in Table I.

A dilution of each reagent that provides saturation of the antigen of interest, low background and enough excess to allow for biological variations in the amount of antigen is selected (Caldwell *et al.*, 1987). Saturation is achieved when, in an antibody dilution test, a plateau is reached where the percentage and intensity of stained cells is constant

TABLE I

Frequently used monoclonal antibodies.

Cluster designation	Name	Isotype	Predominant activity	Source
$CD1_a$	OKT6	IgG_1	Thymocytes	1
CD2	OKT11	IgG_{2a}	E-rosette receptor associated	1
CD3	OKT3	IgG_{2a}	T-cells	1
	Leu4	IgG_1		2
CD4	OKT4	IgG_{2b}	T-helper/inducer	1
	Leu3a	IgG_1	cells	2
CD5	OKT1	IgG_1	T-cells	1
	Leu1	IgG_{2a}		2
CD7	Leu9	IgG_{2a}	T-cells, NK cells	2
CD8	OKT8	IgG_{2a}	T-cytotoxic/suppressor	1
	Leu2a	IgG_1	cells	2
CD9	BA2*	IgG_3	Precursor cells, most non-T-ALL, platelets	3
CD10	J5	IgG_{2a}	Common ALL antigen	4
CD11b	OKM_1	IgG_{2b}	Monocytes, NK cells, granulocytes	1
CD11c	LeuM5	IgG_{2b}	Monocytes, macrophages, hairy cells	2
CD13	My7	IgG_1	Neutrophils, monocytes	4
CD14	My4	IgG_1	Neutrophils,	4
	LeuM3	IgG_{2b}	macrophages	2
CD15	VIMC6	IgM	Granulocytes,	5
	LeuM1	IgM	monocytes	2
CD16	Leu11b	IgM	FC IgG receptor on NK cells and neutrophils	2
CD19	B4	IgG_1	B-cells + B-progenitor cells	4
	Leu12	IgG_1		2
CD20	B1	IgG_{2a}	B-cells	4
CD21	B2	IgM	B-cell subsets, follicular dendritic cells	4
CD22	Leu14	IgG_{2b}	B-cell subset	2
CD24	BA1*	IgM	B-cells, B-progenitor cells	3
CD25	IL_2R_1	IgG_{2a}	IL_2-receptor on activated T-cells	4
CD_w29	4B4	IgG_1	$CD4^+$, CD_w29^+ = T-helper cells	4
CD30	Ki–1	IgG_3	Activation antigen, Hodgkin cells	6
CD33	My9	IgG_{2b}	Monocytes	4

cont'd

TABLE I *cont'd*

Frequently used monoclonal antibodies.

Cluster designation	Name	Isotype	Predominant activity	Source
CD38	OKT10	IgG_1	Thymocytes, activated T-cells, progenitor cells, germinal center B-cells, plasma cells	1
CD_w41	J15*		Platelet gp IIb, IIIa	6
CD_w42	AN51*		Platelet gp Ib	6
CD45	T29/23	IgG_{2b}	Common leukocyte antigen	3
$CD45_R$	2H4	IgG_1	$CD45_{R+}$, $2H4^+$:T suppressor inducer subset	4
—	Leu7	IgM	T-cell and NK-cell subset	2
Anti-transferrin receptor	OKT9	IgG_1	Normoblasts, lymphoblasts, monocytes	1
Anti-HLA-Dr	OKIa	IgG_2	Hemopoietic progenitor cells, B-cell progenitor + B cells	1
—	VIM2*	IgM	Neutrophils, monocytes	5
—	Ki-67*	IgG_1	Nuclear antigen of proliferating cells	6

1. Ortho Diagnostic Systems Inc., Raritan, NJ, USA.
2. Becton Dickinson, Sunnyvale, CA, USA.
3. Hybritech Inc., San Diego, CA, USA.
4. Coulter Immunology, Hialeah, FL, USA.
5. Behringwerke AG, Marburg, FRG.
6. Dakopatts AS, DK 2600 Glostrup, Denmark.

* Clone name instead of commercial name

and independent of antibody concentration (Winchester and Ross, 1986). This saturation can be more precisely determined when the intensity of the immunostaining is measured with a flow cytometer (Caldwell *et al.*, 1987). The working concentrations of the antibodies are usually between 1 and 10 μg/ml. The antibodies are diluted with 5% BSA in PBS. Hybridoma supernatants are used undiluted or at low dilutions.

B. Polyclonal Antibodies

Polyclonal antibodies are still frequently used for the detection of human immunoglobulin heavy and light chains and of terminal deoxynucleotidyl transferase (Tdt), an intranuclear antigen of hemopoietic precursor cells in the bone marrow and thymus. These reagents are titered in the same way as described for monoclonal antibodies.

V. NEGATIVE AND POSITIVE CONTROLS

It is recommended that negative and positive controls are included in each series of tests. Negative control experiments are used to check the presence of non-specific staining. An adequate negative control consists of replacing the specific primary antibody by another antibody produced in the same species but with unrelated specificity. When using polyclonal antisera, a preimmune serum may be used for this purpose. With monoclonal antibodies, normal mouse serum or non-immune mouse ascites is often applied. The latter is obtained from mice implanted intraperitoneally with non-fused, non-secreting plasmatocytoma cells and contains immunoglobulins of all isotypes. Negative control monoclonal antibodies for each isotype are also commerically available. They are used to evaluate the non-specific binding of that isotype to the cells. They are applied in the highest concentration that is used for the specific antibodies. In indirect or multi-step procedures the primary antibody may also be omitted to evaluate the non-specific binding of the other reagents.

Positive controls are used to check the performance of the detection system. Cells such as fresh normal blood leukocytes known to express the antigen are used for this purpose (Caldwell *et al.*, 1987). The number of positive cells and the intensity of the immunostaining can be used as parameters for quality control.

VI. DETECTION OF LEUKOCYTE CELL SURFACE ANTIGENS IN CELL SUSPENSIONS

A. General Staining Procedure

Direct or indirect procedures are used for the staining of cell suspensions. A 25 μl or 50 μl aliquot of the unfixed cell suspension is incubated in a test tube with an appropriate amount of primary antibody for 15–30 min. The cells are then washed twice. One ml of wash buffer is added, the cells are pelleted by centrifugation and the supernatant is removed. In indirect procedures, the pellet is then resuspended in buffer and the

appropriate amount of secondary reagent is added for 30 min to 1 h. The cells are washed again and then processed for examination.

Fixation of the cells before the immunostaining may change the reactivity of the cell surface antigens (Leenen *et al.*, 1985) without improving the morphology of the cells.

The incubations are usually performed on ice or at 4°C and sometimes also in the presence of 0·1% sodium azide to reduce the capping, the endocytosis and the shedding of the immunostaining (Ault, 1986). For routine analysis, however, incubations at room temperature are also adequate.

Leukocyte cell surface antigens in cell suspensions are most often detected with immunofluorescence procedures, but other procedures have also been described.

B. Immun fluorescence Procedures

The cells are labeled with fluorescein (FITC), rhodamin (TRITC) or phycoerythrin (PE) conjugates for immunofluorescence procedures. Fluorochrome conjugates of most of the monoclonal antibodies and of the secondary antibodies are commercially available. In the indirect procedure biotinylated first antibodies and avidin–fluorochrome conjugates may also be used.

For double direct staining of cell surface antigens the monoclonal antibody conjugates may be added simultaneously or sequentially (Thornthwaite *et al.*, 1984; Fleisher *et al.*, 1988). A direct procedure may also be combined with an indirect avidin–biotin procedure (Jackson and Warner, 1986). Double indirect procedures may be performed with two primary antibodies of a different isotype or prepared in a different species and two isotype or species–specific conjugates (Johnson and Holborow, 1986). The two primary or secondary antibodies may be incubated simultaneously.

1. Immunofluorescence Microscopy

The labeled cells are resuspended in 20 µl of a 5% solution of glycerol in PBS at pH 8·0–9·0 and the cells are mounted between microscope glass and coverslip (Janossy, 1981; Hymans *et al.*, 1982). The cells are then identified by their appearance in phase-contrast microscopy (Plate 1, following p. 94). In mixed cell suspensions, like those obtained after red blood cell lysis, this may be rather difficult and time-consuming.

Therefore, mononuclear cell suspensions are most often used. Cells reacting with the antibody show fluorescent patches on their cell surface membrane (Janossy, 1981) (Plate 1). Pre-fixed cells may have a ring-like staining, while dead cells may show a weak diffuse staining over the cytoplasm. This fluorescent staining fades, so long and repeated examinations are difficult. Paraphenylene diamine (1 g/l) (Johnson and Nogueira Aranjo, 1981) or DABCO (Johnson *et al.*, 1982) are effective anti-fading agents when incorporated in the mounting medium. At least two hundred cells have to be examined for an accurate evaluation of the positivity.

Fluorescein conjugates are most frequently used for single staining. Double staining is done with fluorescein and rhodamine conjugates because most fluorescence microscopes are equipped with the appropriate filter systems for these two fluorochromes.

2. Flow Cytometry

The cells are first labeled in test tubes as described above. The labeled cells are resuspended in PBS at a concentration of 10^6 cells/ml. If the suspensions cannot be examined directly they should be stored at 4°C. The cells can also be fixed by adding 1% paraformaldehyde and stored in the dark at 4°C until examination (Jackson and Warner, 1986; Jones *et al.*, 1986). This fixation preserves cellular integrity and fluorescence for up to 5 days.

The same labeling procedure is suitable for immunostaining the cells in microtiter plates. This approach is mainly used when panels of monoclonal antibodies are routinely applied. The plates can be pre-filled with monoclonal antibodies and stored at +4°C or at −70°C. The labeled cells can be examined automatically when the flow cytometer is equipped with a microsampling device (Winchester and Ross, 1986). In flow cytometry the cells in the suspension are identified by their light-scattering properties measured under two angles while the cells pass through the laser beam (Hoffman *et al.*, 1980; Hansen *et al.*, 1982). The forward light scatter correlates with cell volume and the right-angle light scatter is influenced by the granularity of the cells. In a normal leukocyte suspension lymphocytes, monocytes and granulocytes can be distinguished by these characteristics (Fig.1). However it may be difficult to differentiate accurately the erythrocytes from the lymphocytes, or the large granular lymphocytes from the monocytes. In bone marrow the situation is still more complex as only three different populations can be distinguished. The first contains lymphocytes, mature erythroblasts and red blood cells;

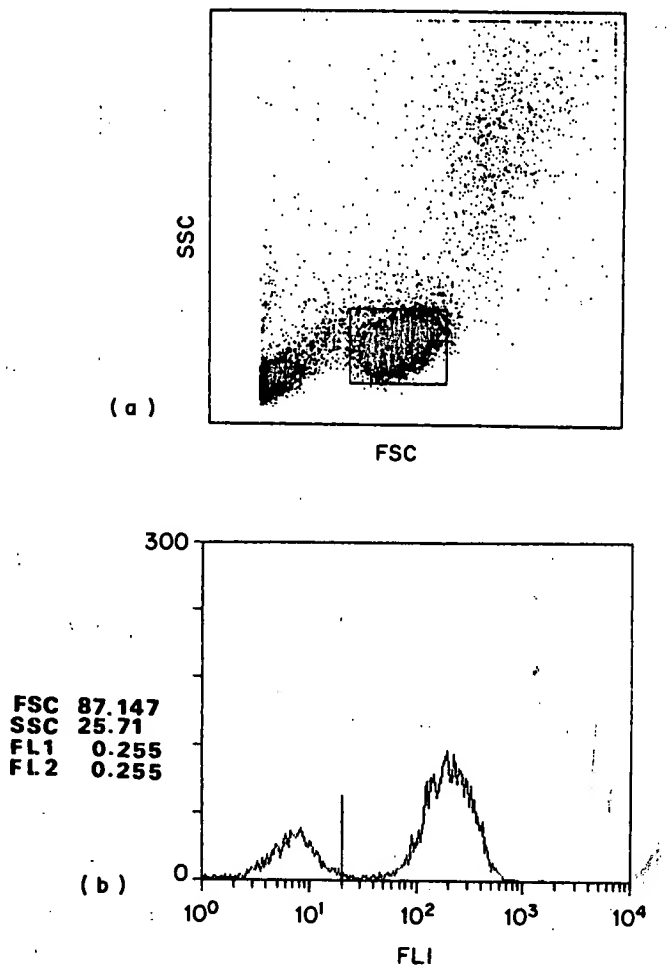

FIG. 1. Detection of cell surface antigens by flow cytometry. A mononuclear cell suspension of normal peripheral blood was labeled with the anti-T-cell monoclonal antibody OKT3 (CD3) and fluorescein-conjugated goat antimouse antibodies. In flow cytometry, the cells were identified by their forward (FSC) and right-angle side scattering (SSC) properties (a). A "gate" was placed around the lymphocytes. The fluorescence intensity (FL1) of the cells in the lymphocyte gate was then plotted on a logarithmic scale against the number of cells showing that intensity (b). Cells with a higher intensity than that of a negative control (cursor) were considered to be positive and were enumerated.

the second population is formed by erythroblasts, monocytes and blasts and the third mainly by granulocytes (Loken *et al.*, 1987). A more accurate identification of the lymphocytes in these samples can be obtained by single or double labeling of the cells with an anti-common-leukocyte antigen antibody (CD45) and/or an antimonocyte antibody (e.g. CD14), an antiglycophorin A antibody to identify the erythroid cells (Shah *et al.*, 1988) or DNA staining (Parker and Haslam, 1988).

A "gate" is placed electronically around the cell population of interest and the fluorescence intensity of the cells in the gate is recorded. This fluorescence intensity is plotted against the relative number of cells showing that intensity (frequency histogram) or against the forward or right-angle light scatter. The latter approach can help to correct the gate setting (Jackson and Warner, 1986). Those cells having a higher fluorescence intensity than that found in a negative control experiment are considered to be positive and are enumerated. Large numbers of cells can be examined within a short time interval so that objective and accurate data are obtained. All data can be stored in the computer and can be reanalysed.

Mononuclear cell suspensions and whole blood after lysis of the red blood cells can be examined by flow cytometry. One-step labeling procedures with fluorochrome conjugated monoclonal antibodies are often applied for this purpose. Most, if not all of the washing steps may then be omitted. In the no-wash procedures mononuclear cells are examined by flow cytometry directly after the incubation with the monoclonal antibody (Caldwell and Taylor, 1986). The presence of this fluorescent conjugate around the cells in the solution produces a small increase of the background fluorescence so that appropriate negative controls should be used. The results generally show a good correlation with those procedures including washing steps (Caldwell and Taylor, 1986). These no-wash techniques are very rapid and may prevent selective cell loss produced by the washing procedures.

With whole blood, the erythrocyte lysis is most often done after the immunostaining. In no-wash procedures the lysis agent is added without removal of the unbound antibody conjugate. The cells should then be examined within 10 min, since prolonged incubation in an ammonium chloride lysis buffer may produce alterations in the cell surface antigens (Caldwell and Taylor, 1986). This may be difficult to perform with a larger number of samples and therefore a washing step is often performed after the red cell lysis

For double labeling, fluorescein and phycoerythrin conjugates are usually used. Both fluorochromes are excited by the same wavelength

(488 nm) but have different emission spectra. With a correct set of filters and an adequate electronic setting of the flow cytometer the two signals are recorded separately. The FITC intensity is usually plotted along the horizontal axis and the PE intensity along the vertical axis. Double labeling on whole blood samples, followed by lysis of the red blood cells, permits the measurement of a large number of samples in a minimum of time (Thorntwaite *et al.*, 1984). The results correlate well with those of immunofluorescence microscopy on mononuclear cells. In addition, double labeling is the only way to identify particular maturation stages or functional leukocyte subpopulations which are characterized by the expression of two or even more antigens (Loken *et al.*, 1987; Shah *et al.*, 1988; Jackson and Warner, 1986; Fleisher *et al.*, 1988).

3. *Double Labeling of Cell Surface and Intracellular Antigens*

Cell surface antigens can be detected together with intracellular antigens by using double labeling procedures. The cell surface antigen is usually detected on unfixed cells in suspension. The cytoplasmic antigen is then stained with the second color after fixation and permeabilization of the cells.

The cell surface phenotype can be studied in relation to the cell cycle by treating the cells, after the surface membrane antigen labeling, with paraformaldehyde and methanol and staining of the nucleic acid with propidium iodide (Noronha and Richman, 1984). Selective staining of DNA or RNA can be obtained after treatment with RNAase or DNAase, respectively. The double-labeled cells can be examined in flow cytometry. The green FITC signal of the membrane antigen and the red signal of the propidium iodide can be examined separately with adequate filter systems. They do not interfere with each other. In this way the cell surface phenotype of activated cells can be studied.

For fluorescence microscopy, cytocentrifuge preparations are usually made after the labeling of the cell surface antigen in suspension. These preparations are then fixed and the intracellular antigen is then detected with a different color. Examples of this approach will be given when staining of intracellular antigens is discussed.

4. *Unwanted Staining*

Unwanted staining may be due to antibody or method non-specificity. We will discuss only those types of unwanted staining which frequently occur with hematological cells. More general information on this subject can be found elsewhere (Van Noorden, 1986).

Damaged or dead cells may bind the reagents non-specifically. These cells occur more frequently in cell smears of imprints but also occur in stored or cryopreserved cell suspensions. They can be removed from these suspensions by centrifugation on a layer of Ficoll-Hypaque.

As already discussed antibodies should be used in the lowest concentration which produce satisfactory immunostaining. Higher concentrations of the reagents than necessary may give an increase in the unwanted background.

Polyclonal primary antibodies may show an unwanted reaction with other antigenic determinants. Such polyclonal antibodies are still frequently used for the study of the immunoglobulin heavy and light chains in B-cells. The specificity of these antibodies can be tested using plasma cells and lymphocytes as biological substrates (Schuit *et al.*, 1981). Primary monoclonal antibodies may also show an unexpected but specific staining with cells in non-related tissues (Mason *et al.*, 1983).

Secondary anti-immunoglobulin antibodies cross-reacting with human immunoglobulins will stain all cells synthesizing immunoglobulin (B-cells) and those which have fixed plasma immunoglobulins on their Fc receptors. Therefore secondary anti-immunoglobulin antibodies absorbed with immobilized human serum or immunoglobulin should preferably be used.

Receptors for the Fc portion of immunoglobulin molecules are found on granulocytes, monocytes, macrophages, natural killer cells, B-cells and activated T-cells. These cells may bind plasma immunoglobulins and this may be responsible for a false high immunostaining when B-lymphocytes are enumerated with anti-immunoglobulin antibodies (Schuit and Hymans, 1980; Ault, 1986). In contrast to B-lymphocytes, these cells have mainly IgG and both kappa and lambda light chains on their surface membrane. Double staining for both light chains may reveal this phenomenon. These cytophilic immunoglobulins can be removed from the cells by incubation in a serum-free medium for 1 h at 37°C and subsequent washing (Ault, 1986). Fixation of the cells before labeling with 0·04% formaldehyde for 10 min may also reduce the detectability of these cytophilic immunoglobulins (Schuit and Humans, 1980).

Cells expressing Fc receptors may also bind immunoglobulin molecules from the reagents, either monoclonal or polyclonal (Gadd and Ashman, 1983; Lawlor *et al.*, 1986; Alexander and Saunders, 1977; Winchester *et al.*, 1975). This phenomenon is mostly expressed on granulocytes and monocytes and is most prominent for IgG_{2a} molecules. It is favored by the presence of immune complexes or aggregates in the reagents. These aggregates may be removed by centrifugation at 8000–30 000 × *g* for 15 min (Jackson and Warner, 1986). The Fc receptors may be saturated by incubating the cells before each labeling step with non-immune serum or

other protein solutions such as bovine serum albumin (Gadd and Ashman, 1983). In addition, Fc receptor binding on leukemic monocytes may be blocked by pre-incubating the cells with aggregated IgG (Lawlor *et al.*, 1986). Goat antibodies show less Fc receptor binding than rabbit antibodies (Alexander and Saunders, 1977). Finally F(ab)$'_2$ fractions of the antibodies may be used to reduce this phenomenon (Winchester *et al.*, 1975). The Fc receptors are inactivated by fixation of the cells and therefore Fc receptor binding is less of a problem in pre-fixed smears.

C. Immun g ld and Immunogold–silver Staining

Colloidal gold was originally introduced as a marker for electron microscopical immunocytochemistry (Faulk and Taylor, 1971). When the density of the marker is sufficiently high, the labeling is also visible in light microscopy (Geoghegan *et al.*, 1978). Based on this principle, immunogold staining methods for the detection of leukocyte cell surface antigens have been developed (Geoghegan *et al.*, 1978; De Waele *et al.*, 1983a). Unfixed cells are incubated with the primary antibody and then with the secondary antibody coupled to colloidal gold particles. Finally smears or cytocentrifuge preparations are made and are counterstained with methyl green, hematoxylin or Giemsa (De Waele *et al.* 1983a; Rosenberg *et al.*, 1984; Wybran *et al.*, 1985). The labeling is visible in brightfield light microscopy as dark granules on the surface membrane of the cells (Plate 2 following p. 94). The labeling is stable and does not change during long or repeated examinations. The results correlate well with those obtained with immunofluorescence procedures. In addition, enzyme cytochemistry can be performed on the immunogold-labeled cells (De Waele *et al.*, 1983b; Crockard and Catovsky, 1983; Bergroth *et al.*, 1983). This combination can be used to improve the cell identification or to determine the cytochemical profile of a particular leukocyte subset. The gold particles reflect incident light and are visible as bright granules in darkfield and epipolarization microscopy (De Waele *et al.*, 1983a; De Mey, 1983). Immunogold-stained lymphocytes can also be enumerated with the flow cytometer (Bohmer and King, 1984; Festin *et al.*, 1987). They show an increase of the right-angle light scatter while the forward light scatter remains unchanged. This signal does not interfere with that of fluorescein and phycoerythrin so that colloidal gold can be used together with these fluorochromes for triple labeling of cell surface antigens in single laser flow cytometry. Immunogold staining has also been combined with immunofluorescence in a multiple labeling procedure for microscopy (Van Dongen *et al.*, 1985) and with an immunoperoxidase

technique for the detection of terminal deoxynucleotidyl transferase (Tdt) in the nucleus of acute leukemia cells (Tavares de Castro *et al.*, 1984).

The visibility of immunogold staining can be increased by silver enhancement (Holgate *et al.*, 1983). Cytocentrifuge preparations of immunogold-labeled cells are treated with a physical developer. In this medium concentric layers of metallic silver are deposited around the gold particles. This increases their diameter and their visibility in light microscopy. A dense dark labeling is obtained which permits the use of Romanovsky counterstains such as May–Grunwald–Giemsa or Wright–Giemsa (Plate 3 following p. 94) (Romasco *et al*, 1985; De Waele *et al.*, 1986a). The morphology of the labeled cells is now comparable to that seen in smears made for routine morphological examination. The good morphology obtained makes immunogold–silver staining an ideal technique for the study of the cell surface phenotype of particular morphological cell types in mixed cell suspensions, e.g. bone marrow aspirates or lymph node cell suspensions (Plate 4, following p. 94) (De Waele *et al.*, 1986b, 1988b). The labeling efficiency of the method can be increased by an examination of the preparations in darkfield and epipolarization microscopy (Plate 5, following p. 94) (De Waele *et al.*, 1988a).

D. Immunoenzyme Techniques

Only a few reports are available in which immunoenzyme techniques are used for the detection of cell surface antigens on leukocytes in suspension. Yam *et al.* (1983) described an indirect alkaline phosphatase method for this purpose. Unfixed cells are incubated with monoclonal antibodies and then with alkaline phosphatase conjugated goat antimouse antibodies. Cytocentrifuge preparations were made. They were fixed and the alkaline phosphatase was revealed. The preparations were counterstained with hematoxylin. Positive cells possessed a bright red granular deposit on the surface membrane. The endogenous alkaline phosphatase in the neutrophils was effectively inhibited by levamisole and the relatively low pH of the medium. Stained slides had to be kept unmounted as the staining product was not stable in mounted slides. When the whole procedure could not be carried out on the same day, the detection of certain antigens was less reliable.

In a more recent study, Yam *et al.* (1987) preferred the indirect immunoalkaline phosphatase method to other immunoenzyme methods for staining the cells in cerebrospinal fluid. It was found that immunoperoxidase methods showed more background staining due to endogenous peroxidase. The more sensitive alkaline phosphatase antialkaline phospha-

tase (APAAP) and the avidin–biotin–alkaline phosphatase complex (ABC–AP) methods contained more steps and gave a high cell loss during the procedures.

E. Immun bead Methods

Plastic beads of different sizes coupled to antibodies have also been used for the detection of cell surface antigens (Baran and Parker, 1985; Mirro and Stass, 1985; Tomaszewski *et al.*, 1986; Bourel *et al.*, 1988; Homans *et al.*, 1986; Brinckman *et al.*, 1988). Beads with different colors visible in brightfield light microscopy (Baran and Parker, 1985) or fluorescent beads (Mirro and Stass, 1985; Tomaszewski *et al.*, 1986; Bourel *et al.*, 1988; Homans *et al.*, 1986) have been applied for this purpose. The labeled cell suspensions were examined between microscope glass and coverslip or in counterstained cytocentrifuge preparations. Positive cells showed numerous beads around their surface membrane. A good correlation was obtained with immunofluorescence methods. Double labeling of two cell surface antigens in different cells was possible with beads of different colors (Baran and Parker, 1985; Homans *et al.*, 1986). Monocytes showed an active binding and phagocytosis of the beads. This could be blocked by pre-fixation of the cells (Mirro and Stass, 1985; Tomaszewski *et al.*, 1986). With double labeling methods the monocytes could also be identified because they contained beads of both colors (Baran and Parker, 1985; Homans *et al.*, 1986).

In one study, immunomagnetic beads were used and the labeled cells were then isolated with a magnet (Brinckman *et al.*, 1988). The cell membranes were lysed and the cell nuclei in the isolated suspensions were counted after counterstaining with acridine orange. In this way, absolute values for the lymphocyte subsets were obtained.

So far, only a limited number of leukocyte cell surface antigens have been detected in this way.

VII. DETECTION OF LEUKOCYTE CELL SURFACE ANTIGENS IN CELL SMEARS

A. Immun enzyme Techniques

Immunoenzyme techniques are mainly used for the detection of leukocyte cell surface antigens in smears (Moir *et al.*, 1983). Smears or cytocentrifuge preparations of the cell suspension are air dried for 2 to 24 h at room

temperature before labeling. Preparations taken from the freezer must be allowed to reach room temperature before the parafilm or aluminum foil is removed, because condensation of water from the air on the cold microscope slides could destroy the structure of the unfixed cells. The preparations are then fixed to preserve cellular morphology and to prevent cell loss during the labeling procedure. Various fixatives, mainly based on acetone, methanol and/or formol, have been applied for this purpose. In general, stronger fixation gives a better morphology but a weaker immunostaining. The best morphology is obtained with a phosphate-buffered 10% formol–45% acetone (pH 6·6) fixation for 30 s at room temperature (Moir *et al.*, 1983; Mason *et al.*, 1977; Erber *et al.*, 1986; Wong *et al.*, 1986) or at 4°C (Li *et al.*, 1984; Yam *et al.*, 1987). With acetone:methanol (1:1, 4°C, 90 s) or acetone:methanol:formol (19:19:1, 4°C, 90 s) the morphology is less good but the immunostaining is intense. These mixtures are the fixatives of choice for the alkaline phosphatase-antialkaline phosphatase technique (Mason *et al.*, 1986; Erber *et al.*, 1987). Acetone (10 min, 6°C) gives the strongest immunostaining, but also a bad morphology of the leukocytes and a lysis of the red blood cells (Mason *et al.*, 1986). The preparations should be air dried after acetone fixation. With all other fixatives the preparations are rinsed in buffer after fixation and the immunostaining is then performed. The zone to be stained is encircled with a diamond pen. The slides are incubated horizontally in a moist chamber and the reagents are put on top of the encircled zone. The immunostaining is usually performed with indirect (Banks *et al.*, 1983; Li *et al.*, 1984; Wong *et al.*, 1986; Lowenthal and Marsden, 1986; Yam *et al.*, 1987) or multi-step procedures (Moir *et al.*, 1983; Mason *et al.*, 1977; Giorno, 1983; Sandhaus *et al.*, 1984; Erber *et al.*, 1986; Aratake *et al.*, 1988; Yam *et al.*, 1987) with horseradish peroxidase (Mason *et al.*, 1977; Banks *et al.*, 1983; Giorno, 1983; Sandhaus *et al.*, 1984) or alkaline phosphatase (Moir *et al.*, 1983; Li *et al.*, 1984; Erber *et al.*, 1986; Wong *et al.*, 1986; Yam *et al.*, 1987) as marker. The multi-step procedures use pre-formed enzyme–antienzyme antibody complexes (Moir *et al.*, 1983; Mason *et al.*, 1977; Erber *et al.*, 1986, Yam *et al.*, 1987) or avidin–biotin–enzyme complexes (Giorno, 1983; Sandhaus *et al.*, 1984; Aratake *et al.*, 1988; Yam *et al.*, 1987) for the detection.

The horseradish peroxidase is then developed with 3,3′-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) and the alkaline phosphatase with a naphthol AS phosphate, naphthol AS-MX phosphoric acid or naphthol AS-Bi phosphate as substrate and Fast Red Violet LB salt, Fast Red TR or New Fuchsin as coupler. The preparations are usually counterstained with hematoxylin, which gives a good nuclear morphology. Although Fc receptor binding is less in pre-fixed smears than in unfixed

cell suspensions, a pre-incubation with serum or protein solutions is often done to prevent non-specific binding of the reagents occurring (Giorno, 1983; Sandhaus *et al.*, 1984; Aratake *et al.*, 1988). Alternatively, these proteins can also be added during incubations with the antibody.

With these immunoenzyme methods the positive cells have a diffuse or more granular brown (DAB) or red staining (AEC, alkaline phosphatase) all over the surface membrane of the cell (Plate 6, following p. 94). As the cells are fixed before labeling, capping, endocytosis and shedding of the immunostaining are not possible. With antibodies giving strong immunostaining the reaction product is already visible at low magnification. Weak reactions may be masked by the hematoxylin counterstaining. The intensity of the immunostaining of indirect techniques may vary significantly between antibodies, even between those belonging to the same CD group. The CD3 antibody Leu4, for instance, gives a strong staining with the indirect alkaline phosphatase method while OKT3 produces ony a weak staining (Li *et al.*, 1984).

In a recent study on cerebrospinal fluid cells, Yam *et al.* (1987) preferred the alkaline phosphatase–antialkaline phosphatase (APAAP) technique for the labeling of cell smears. This technique was more sensitive than the indirect techniques. It had approximately the same sensitivity as the avidin–biotin–alkaline phosphatase complex technique (ABC-AP), but the latter showed considerable background staining which was difficult to control. With immunoperoxidase techniques the endogenous peroxidase activity in granulocytes and monocytes caused a background staining. Attempts to eliminate this activity invariably led to a drastic reduction or abolition of the surface antigens. Giorno (1983), however, could block the endogenous peroxidase by a treatment with 0·3% H_2O_2 for 5 min before the labeling without any reduction of the immunostaining. Sandhaus *et al.* (1984) treated the cells with 0·3% H_2O_2 in methanol for 30 min just before the incubation with the ABC complex, but the endogenous peroxidase activity still persisted in some neutrophils and eosinophils. This interfered with the detection of low numbers of positive cells in the samples. In a recent article Li *et al.* (1987) also reported that treatment with 0·3% hydrogen peroxide and 0·1% sodium azide before the labeling adequately blocks the endogenous peroxidase activity of neutrophils and monocytes while the immunostaining is not affected. The eosinophilic peroxidase persisted but was much less revealed when AEC was used as a substrate at pH 5·2 instead of DAB at pH 7·4, since the optimal pH for eosinophilic peroxidase is between 6 and 8.

Intracellular enzymatic activities are less problematical when using the APAAP technique. The endogenous alkaline phosphatase activity in granulocytes is adequately blocked by the addition of 0·01 M levamisole to the substrate solution. The calf intestinal alkaline phosphatase which

is used in the APAAP complex is insensitive to this inhibitor. For weak antigens the staining intensity obtained with the APAAP procedure can be enhanced by repeating the second and third incubation steps of the procedure for 10 min each. Overstaining may lead to the formation of a coarse granular brown product on and between the cells (Mason, 1985).

Immunolabeling of cells in smears and cytocentrifuge preparations is particularly interesting for samples such as cerebrospinal fluid with small numbers of cells (Yam *et al.*, 1987) or blood smears prepared from a fingerprick (Mason, 1985). Touch imprints from biopsies of lymphoid tissues can also be stained, but the presence of many damaged cells (Banks *et al.*, 1983) and of plasma protein between the cells may cause a lot of background. In addition, surface immunoglobulin cannot be studied in these preparations. A good nuclear morphology is obtained so that mixed cell suspensions such as bone marrow can be examined (Moir *et al.*, 1983; Sandhaus *et al.*, 1984; Erber *et al.*, 1986; Lowenthal and Marsden, 1986). This also reduces the need for intensive cell purification before the labeling procedure. The APAAP method gives a strong immunostaining that is visible even at low magnification so that rapid scanning of the preparations is possible. The preparations may be stored before labeling for at least 1 week at room temperature and 1 year in the freezer (Giorno, 1983).

The detection of cell surface antigens with immunoenzyme methods in cell smears can be combined with the autoradiographic detection of DNA synthesis (Schneider and Wachner, 1986; Konttinen *et al.*, 1988). Therefore unfixed cells are incubated at 37°C with tritiated thymidine which is incorporated in the nuclei of the cells synthesizing DNA. Then cytocentrifuge preparations are made. They are fixed and the cell surface antigen is stained with an immunoenzyme technique. Immunoperoxidase (ABC or PAP) and immunoalkaline phosphatase techniques (APAAP) have been used for this purpose. Then a photographic emulsion is applied for the autoradiographic study. Cells which have incorporated thymidine show dark silver grains over the nucleus. This signal does not interfere with the enzymatic reaction product on the cell surface membrane. According to Schneider and Wachner (1986) the red reaction product of the APAAP method gives a better contrast with the dark silver grains than the brown DAB product of the DAB reaction. In this way the cell surface phenotype can be studied in relation to the cell cycle.

B. Immunofluorescence and Immunogold–silver Staining

Cell surface antigens in cell smears have also been detected with indirect immunofluorescence methods (Lovat *et al.*, 1987; Joly *et al.*, 1986) and

with immunogold–silver staining (De Waele *et al.*, 1986b). With the latter technique a stable staining and a good morphology is obtained (Plate 7, following p. 94).

VIII. DETECTION OF INTRACELLULAR ANTIGENS

Intracytoplasmic and intranuclear antigens which are frequently detected for diagnostic purposes include cytoplasmic immunoglobulin, the CD3 and CD22 antigens, terminal deoxynucleotidyl transferase (Tdt), the proliferating cell nuclear antigen detected by the Ki-67 monoclonal antibody, and finally bromodeoxyuridine incorporated during DNA synthesis.

A. Cyt plasmic Immunoglobulin

Cytoplasmic immunoglobulin is mainly present in plasma cells of bone marrow and lymphoid tissue. It is usually stained in cytocentrifuge preparations. For immunofluorescence microscopy a pre-fixation with 5% glacial acetic acid in absolute ethanol for 15 min at −20°C has been used (Hymans *et al.*, 1969). This treatment sufficiently permeabilizes the cell to allow penetration of the antibodies. A one-step labeling procedure, using polyclonal anti-human immunoglobulin antibodies, is frequently applied. The preparations are incubated with these reagents for 30 min at room temperature. The cells are not counterstained and the preparations are mounted with PBS-buffered glycerol. Plasma cells and lymphoplasmatocytic cells generally show a strong fluorescence of the whole cytoplasm (Plate 8, following p. 94). A cytoplasmic fluorescence in lymphocytes is rare. A localized immunostaining can be seen in chronic lymphocytic leukemia cells containing intracytoplasmic crystalline inclusions (Peters *et al.*, 1984). Without counterstaining, cell identification in the preparations is difficult. Phase-contrast optics are of limited value because the cells are flattened on the microscope glass.

Double staining with FITC and TRITC conjugates is frequently carried out in order to obtain the relative proportions of plasma cells synthesizing kappa or lambda light chains. The predominance of one of these light chains is an argument for the existence of a monoclonal cell proliferation, similar to multiple myeloma (Hymans *et al.*, 1969; Van Camp *et al.*, 1981) or Waldenstrom-like lymphoma (De Waele *et al.*, 1981). A monoclonal light chain secreting plasma cell proliferation in the bone marrow is also found in patients with primary amyloidosis (Thielemans *et al.*, 1982).

However not all positivity in these fixed cytocentrifuge preparations is due to cytoplasmic immunoglobulins (Schuit *et al.*, 1984). Cell surface immunoglobulin of B-lymphocytes resists fixation and is stained as a confluent ring. True cytoplasmic immunoglobulin in peripheral blood lymphocytes is rare and is only found in cells with abundant cytoplasm.

Double labeling of a cell surface antigen and intracytoplasmic immunoglobulin is feasible (Van Camp *et al.*, 1982). The cell surface antigen is labeled in suspension with FITC conjugates. Cytocentrifuge preparations are made and fixed with acetone (10 min at −20°C). Cytoplasmic immunoglobulin is then stained, as described above, with the TRITC conjugates. In this way the cell surface phenotype of plasma cells can be examined (Van Camp *et al.*, 1982; Lokhorst *et al.*, 1987). (Plate 9 following p. 94).

Both surface and cytoplasmic immunoglobulins can also be detected when the cytocentrifuge preparations are fixed with buffered formol–acetone, as described for cell surface antigens, and are then stained with the PAP (Mason *et al.*, 1977) or the APAAP technique (Plate 10, following p. 94) (Moir *et al.*, 1983).

The cytocentrifuge preparations can be stored at −20°C for at least one year before the detection of cytoplasmic immunoglobulin.

B. CD3 and CD22 Antigens

The CD3 antigen is present on the surface membrane of 60% of thymocytes and all peripheral blood T-cells (Foon and Todd, 1986). Recently the CD3 antigen was also demonstrated in the cytoplasm of 95% of thymocytes and in all T-cell acute lymphoblastic leukemias, also in those where surface membrane CD3 was absent (Van Dongen *et al.*, 1988; Campana *et al.*, 1987).

For cytoplasmic CD3 staining, the cytocentrifuge preparations were fixed in absolute acetone for 5 to 10 min at 20°C (Campana *et al.*, 1987) or with 5% acetic acid (v/w) in ethanol for 15 min at −20°C (Van Dongen *et al.*, 1988). An indirect immunofluorescence procedure was used. In these preparations both the surface membrane antigens, if present, and the cytoplasmic reactivity were detected. The surface membrane staining had either a ring-like pattern, or was diffuse, while the cytoplasmic staining was localized around the nucleus, in the form of dots or irregular filaments. When the surface membrane staining was very bright, it was difficult to detect a weak cytoplasmic staining. Not all anti-CD3 antibodies were able to detect the cytoplasmic activity (Van Dongen *et al.*, 1988). The two-color labeling of a cell surface antigen with cytoplasmic CD3 has also been described (Campana *et al.*, 1987; Van Dongen *et al.*, 1988).

The cell surface antigen was labeled on unfixed cells in suspension. Then cytocentrifuge preparations were made and the cytoplasmic CD3 was detected with the second color as described above. The surface membrane staining on the unfixed cells in suspension has a more patchy appearance than that stained on the fixed cytocentrifuge preparations.

The cytoplasmic CD3 activity has also been detected with flow cytometry (Mirro *et al.*, 1987). Therefore the cells were treated before labeling with 5 μg/ml lysolecithin in PBS, pH 7·2 for 3 min at 4°C. This permeabilized the cell membranes to allow the penetration of the antibodies.

Cytoplasmic CD3 has also been stained in cytocentrifuge preparations fixed with acetone for 3 min at 4°C, and an ABC–peroxidase technique (Mirro *et al.*, 1987).

The cytoplasmic expression of CD22 antigen appears to be one of the earliest markers of B-cell ontogeny (Dorken *et al.*, 1987). This marker appears in the cytoplasm of pre-pre-B-cells. It is present on the cell surface of resting B-cells and is lost with cellular activation. It has been detected in cytocentrifuge preparations with the same APAAP method described earlier for the detection of cell surface antigens (Dorken *et al.*, 1987). (Plate 10, following p. 94).

C. Terminal D oxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (Tdt) is a DNA polymerase present in the nucleus of cortical thymocytes and of a small population of bone marrow cells. It is also present in the cells of the majority of acute lymphoblastic leukemia patients, in lymphoid blast crisis of chronic myeloid leukemia, in a minority of acute myeloid leukemias and in a number of non-Hodgkin lymphomas (Bollum, 1979).

Tdt has been detected with an indirect immunofluorescence procedure in cytocentrifuge preparations fixed with absolute methanol at 4°C for 30 min. A polyclonal rabbit anti-calf Tdt, showing a cross-reaction with human Tdt, was used for this purpose (Bollum, 1979; Dunbar *et al.*, 1985). A bright reticulated nuclear fluorescence was seen. The fixed preparations could be stored for at least 4 weeks at −30°C, before labeling.

Van Dongen *et al.* (1986) performed double labeling of T-cell surface antigens and Tdt. The surface membrane antigens were labeled on unfixed cells in suspension. Tdt was stained in fixed cytocentrifuge preparations of the cell suspension. This double staining may increase the detection sensitivity of residual T-ALL leukemic cells in extrathymic sites.

Immunoenzymatic staining of Tdt has also been performed with both the PAP and the avidin–biotin–peroxidase technique. Permanent

preparations with a good cell morphology were obtained (Stass *et al.*, 1982; Fetterhoff *et al.*, 1985; Hecht *et al.*, 1981). The interpretation of the results, however, was difficult in samples where the endogenous peroxidase was not completely blocked. This problem did not occur when alkaline phosphatase was used as marker. The multi-step APAAP technique gave more intense staining than the indirect immunoalkaline phosphatase technique (Erber and Mason, 1987). Monoclonal anti-Tdt antibodies gave the same staining patterns as polyclonal antibodies but with a lower intensity. With the APAAP technique a coarse granular labeling of the nuclei and a diffuse cytoplasmic staining were seen. Cells in mitosis showed extensive cytoplasmic staining and the megakaryocytes were also positive. The significance of the cytoplasmic Tdt staining remains uncertain.

D. Proliferating Cell Nuclear Antigens

The Ki-67 monoclonal antibody detects an antigen in the nucleus of proliferating cells. This antigen can be detected in cytocentrifuge preparations with immunoenzyme techniques. Gerdes *et al.* (1986) stained this antigen with a three-step immunoperoxidase technique, together with the Ki-1 (CD30) surface membrane antigen, detected with the APAAP method. The Ki-1 monoclonal antibody was originally raised against Hodgkin cells but the antigen is also found on the surface membrane of large lymphoid cells in reactive lymphoid tissue at the rim of the B-cell follicles. Gerdes *et al.* (1986) found that nearly all Ki-1 positive lymphocytes were Ki-67 positive and thus were proliferating cells. He assumed that the Ki-1 positive lymphomas, including Hodgkin's disease, are neoplasms of activated lymphoid cells that might be derived from those normal perifollicular lymphocytes.

Lokhorst *et al.* (1987) performed the same double staining technique to study the Ki-67 nuclear antigen in a particular subset of plasma cells, characterized by a spot-like localization of the cytoplasmic immunoglobulin. This subset of plasma cells, found in the bone marrow of multiple myeloma patients, was Ki-67 positive and thus was highly proliferative.

Using an avidin–biotin–peroxidase technique on cryostat sections of lymphoid tissues, Grogan *et al.* (1988) showed that a high proliferative activity, as defined by a nuclear Ki-67 expression in more than 60% of the malignant cells, is associated with a poor outcome of diffuse large cell lymphoma.

Proliferating cell nuclear antigen (PCNA/cyclin) was detected with flow cytometry in cells labeled in suspension after treatment with 500 μg/ml lysolecithin in 1% paraformaldehyde for 5 min, followed by absolute

methanol for 10 min at −20°C (Kurki *et al.*, 1988). These conditions induced some cell shrinkage, however. The staining of the nuclear antigens with FITC-labeled antibodies was combined with DNA staining by propidium iodide to study the expression of the antigen in relation to the cell cycle.

E. Brom deoxyuridine Uptake

When bromodeoxyuridine (BrdU), a thymidine analogue, is incubated with unfixed cells at 37°C, it is incorporated in cells synthesizing DNA. These cells in the S phase of the cell cycle can then be enumerated with antibodies directed against bromodeoxyuridine. In this way the proliferative capacity of the cells can be determined. This method gives results comparable to those of the tritiated thymidine labeling but is easier and more rapid to perform (Lokhorst *et al.*, 1986).

The cells in preparation for flow cytometry are fixed in suspension after the BrdU uptake, with 70% methanol for 30 min at 0°C (Dolbaere *et al.*, 1983). As the monoclonal antibodies against BrdU only detect the antigen in single-stranded DNA, the nuclear DNA has to be denatured before the labeling. This can be done with strong acidic (HCl) or basic (NaOH) solutions. After neutralization of the acid or base, the monoclonal anti-BrdU antibody is added for 30 min at room temperature. A direct or indirect immunofluorescence procedure may then be used. Tween 20 is added to the incubation media to achieve a better permeabilization of the cells. After a washing procedure, the cells are examined with the flow cytometer. The cells can be counterstained with propidium iodide allowing a simultaneous evaluation of the total cellular DNA content.

For microscopic examination, cytocentrifuge preparations are made after the BrdU uptake. Fixation, DNA denaturation and labeling are done on these preparations. Lokhorst *et al.* (1986) used a double immunofluorescence procedure to examine the BrdU uptake in plasma cells which were identified by the TRITC staining of cytoplasmic immunoglobulin. The sensitivity of this method was similar to that of the autoradiographic method with tritiated thymidine. However the morphology of the plasma cells in phase-contrast microscopy was not optimal because of the strong fixation and DNA denaturation.

Van Furth and Van Zwet (1988) obtained better morphology by using a 25% glacial acetic acid/absolute ethanol fixative, and a DNA denaturation with lower concentrations of HCl followed by heating and cooling. They used a three-step avidin–biotin–peroxidase technique and counterstained with Giemsa.

Margaud *et al.* (1988) visualized the BrdU uptake with the APAAP method in cytocentrifuge preparations fixed for 10 min in absolute ethanol followed by a DNA denaturation with formamide.

Recently a monoclonal anti-BrdU was described which contains a DNase secreted by mycoplasma contaminating the hybridoma (Gonchoroff *et al.*, 1985; Greipp *et al.*, 1985) (Plate 11, following p. 94). With this antibody, no additional DNA denaturation step has to be performed. However Van Furth and Van Zwet (1988) obtained lower percentages of positive cells with this antibody than with the other antibodies and DNA denaturation.

IX. CONCLUSIONS

The abundance of recent publications indicates that immunocytochemistry, using monoclonal and polyclonal antibodies, is a valuable technique in clinical hematology. This chapter has reviewed diagnostic hematology. There is now also interest in the application of immunocytochemistry for basic research into the understanding of normal cell development and in the malfunctioning disease states. In consequence, the techniques used are becoming more varied and encompass both histology and electron microscopy. Much of the histological research is centred around the use of sections either frozen, embedded in paraffin or embedded in plastic. There appears to be a heavy bias towards immunoenzyme techniques, both the immunoperoxidase (Archimbaud *et al.*, 1987) and the alkaline phosphatase (Ormans and Schaffer, 1985) techniques being used. Colloidal gold probes in conjunction with silver enhancement techniques (Holgate *et al.*, 1983), however, are now becoming popular. The majority of electron microscopical studies, in the biological sciences as a whole, employ colloidal gold probes. These are used for both transmission (Cramer *et al.*, 1988) and scanning (Soligo *et al.*, 1987) electron microscopy and are proving extremely versatile for both double labeling and quantification studies.

In conclusion there is ample evidence to suggest that immunocytochemical techniques will continue to be important in both clinical and research hematology, combining the unique quality of visualizing a particular cell and, at the same time, examining the chemistry of that same cell.

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Application of *In Situ* Hybridization with Radioactive Nucleotide Probes to Detection of mRNA in the Central Nervous System

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Separation of Human Blood and Bone Marrow Cells

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To my family, and especially to my mother and to the memory of my late father.

May God bless them.

Chapter 3

Isolation of Blood Cells by Centrifugation on Percoll Gradients

1. INTRODUCTION

In recent years centrifugation has proved to be one of the most useful techniques for the fractionation of biological particles. Different types of cells can be separated according to difference in buoyant density which is termed 'isopyknic' centrifugation, or according to variation in size as in the case of 'rate-zonal' centrifugation. Stokes' law describes the sedimentation rate (V) of a sphere with a diameter d in a gravitational field g:

$$V = \frac{d^2(\rho_p - \rho_l)\, g}{18\eta}$$

where ρ_p and ρ_l are the respective densities of the spherical particles (e.g. cells) and liquid (gradient material), and η is the viscosity of the liquid. Stokes' law adequately describes the behaviour of cells during isopyknic centrifugation, in which separation is effected on the basis of density. In addition, the above equation applies to velocity sedimentation in which separation is on the basis of size.

1.1 ISOPYKNIC CENTRIFUGATION (EQUILIBRIUM DENSITY CENTRIFUGATION)

The cells are sedimented on the basis of density through an increasingly dense medium (continuous density gradient) during the application of a gravitational force (g) by centrifugation. The density range of the gradient medium encompasses all densities of the cells present in the sample. The cells float in an equilibrium position after reaching a point where their density equals that of the medium. At this

position (ρ_p and ρ_l) = 0, therefore sedimentation rate (V) = 0. Cells with a density greater than that of the gradient sediment to the bottom of the tube. The density gradient is formed from substances which will not perturb osmotic balance or ionic equilibria. These include very high molecular weight polymers of low osmolality. The cells are either applied to the top of the gradient or are mixed into the gradient medium with the dense solution, which helps to minimize streaming of the cells. Reproducibility requires careful temperature control and, in order to prevent perturbation, the centrifuge is operated with the brake in the 'off' position.

1.2 ISOPYKNIC CUSHIONING

A cell suspension is layered onto a high-density medium, the density of which varies according to the type of cells that are to be isolated. The basis of the cell separation is the same as the isopyknic centrifugation, but the cells collect at the interface between the media. A gravitational force is applied by centrifugation and at equilibrium those cells having a density lower than that of the cushion ($\rho_l > \rho_p$) are collected at the cushion/medium interface, whereas the more dense cells are found in the pellet ($\rho_l < \rho_p$) at the bottom of the tube. A discontinuous gradient may include a single density layer (single-step cushioning) or may be constructed from several cushions, one on top of the other, starting with the highest density at the bottom of the tube to the lowest density at the top. The cells are collected at the appropriate interface between medium of $\rho_l < \rho_p$ and medium of $\rho_l > \rho_p$.

1.3 DENSITY GRADIENT MEDIUM (PERCOLL)

Density gradient centrifugation has for many years been used for the separation of cells. The restrictions placed on using this technique have mostly been due to the physical properties of the gradient materials available for use. At the density required for optimum resolution, the conditions created by many media are far from the physiological norm, thus leading researchers to compromise between resolution (degree of purification) and retaining the biological integrity of the cells.

Isopyknic centrifugation has been facilitated by the introduction of Percoll (Pharmacia Fine Chemicals). Percoll is a colloidal suspension of silica particles coated with polyvinylpyrrolidone (PVP) which serves as a stabilizer and avoids silica toxicity to the cells. The suspension can be made isotonic and adjusted to physiological pH before preparation of density gradients.

Pure colloidal silica solutions (Ludox-HS; Pharmacia Fine Chemicals) was first reported as being useful for cell separation by Mateyko and Kopac (1963), and was then systematically evaluated by Pertoft and

Laurent (1968, 1977) and by Wolff (1975). It was found that unmodified silica solutions were unstable in the presence of salt at physiological pH and were toxic to cells. This instability and toxicity was decreased by the addition of polymers such as PVP, dextran and polyethylene glycol. However, a large excess of free polymer was required and this not only increased the osmolality and viscosity but was also difficult to remove from suspensions. Percoll (modified colloidal silica MCS, Pertoft and Laurent, 1977) was then developed to overcome these problems. Each silica particle has a 15–30 nm diameter and is irreversibly coated with PVP (Pertoft et al, 1978). Percoll suspensions are supplied at a density of about $1{\cdot}13 \pm 0{\cdot}005$ g/ml and can form gradients in the range 1·0 to 1·3 g/ml, which covers a density range sufficient for isopyknic separation of all human blood cells. Percoll has a very low osmotic pressure of 15–20 mmol/kg H_2O and can therefore give a density gradient virtually iso-osmotic throughout. It also has a low viscosity of 10 ± 5 centipoises (cP) at 20 °C which allows an equilibrium to be obtained rapidly. Percoll is easily removed from cells (Bergman et al, 1977), it can be sterilized by filtration and is completely non-toxic to cells (Pertoft et al, 1977; Kurnick et al, 1979). All of these characteristics make Percoll an excellent gradient medium for the separation of cells. Although Ficoll (Pharmacia Fine Chemicals), has been used for many years to separate blood cells and particularly peripheral blood mononuclear cells (Böyum, 1968), a major advantage of Percoll is its ability to generate a continuous density gradient when centrifuged at high *g* values.

Continuous density gradients of Percoll have previously been used by many workers for the separation of monocytes, lymphocytes and neutrophils (Kurnick et al, 1979; Gmelig-Meyling and Waldmann, 1980; Segal et al, 1980; Giddings et al, 1980). Modification of the starting density enables a good recovery of highly purified lymphocyte subpopulations (Ali et al, 1982). Isolated cells exhibit a high viability (Pertoft et al, 1977; Feucht et al, 1980).

Discontinuous Percoll gradients have been used to separate monocyte and lymphocyte subpopulations (Gutierrez et al, 1979; Ulmer and Flad, 1979). Kurnick et al (1979) also separated low density B-lymphocytes from T-lymphocytes. A complete up-to-date list of references of research carried out using Percoll may be requested from Pharmacia Fine Chemicals.

2. PREPARATION OF PERCOLL SOLUTIONS OF DIFFERENT DENSITIES

Percoll solutions are required at a range of starting densities since cells differ in their buoyant densities. Silica-containing solutions usually

give a pellet at the bottom of the centrifuge tube, and deposits of silica on the walls of tubing used for separation. These deposits should be removed before drying by washing thoroughly with water immediately after use.

2.1 DIRECT DILUTION OF STOCK PERCOLL

The stock suspensions of Percoll may be diluted directly from the bottle to give the desired density (working solution) as described below.

Materials

i. Stock Percoll (Pharmacia Fine Chemicals): mix before use.
ii. 1·2M NaCl: 8% (w/v) solution.

Procedure

a. Choose the final volume (V) of Percoll solution of the desired density. Place a volume of 1·2M NaCl equal to one-tenth of the final volume V, into a sterile container. The volume of purchased Percoll to be added to this solution is calculated by the following formula (work from Pharmacia Fine Chemicals reproduced by kind permission):

$$V_0 = V \frac{\rho - 0{\cdot}1\rho_{10} - 0{\cdot}9}{\rho_o - 1}$$

where: V_0 = volume of stock Percoll (from the bottle), ml
V = volume of the final working solution, ml
ρ = desired density of the final solution, g/ml
ρ_o = density of stock Percoll (printed on the bottle), g/ml
ρ_{10} = density of 1·2M NaCl = 1·056 g/ml

b. Make up to the final volume with distilled water.
c. Measure the density using the refractometer (Section 3).
d. Measure the osmolality of the working solution. This should be in the range 280–300 mmol/kg H_2O.
e. A graph identical to the one shown in *Fig.* 3.2 (p. 61) may be drawn to relate the volume of purchased Percoll to the final density.

Example:

To prepare 100 ml of working Percoll solution of density 1·077 g/ml in 1·2M NaCl, place 10 ml of 1·2M NaCl into a container. The calculated volume of stock Percoll (V_o) to add is:

$$V_0 = 100 \frac{1{\cdot}077 - (0{\cdot}1 \times 1{\cdot}056) - 0{\cdot}9}{1{\cdot}13 - 1}$$

= 54·9 ml (if stock Percoll density is 1·13 g/ml). Make up to 100 ml with distilled water.

Comment

The osmolality of the working solution prepared using 1·5M NaCl (9% w/v, as suggested by Pharmacia Fine Chemicals) was 325–330 mmol/kg H_2O. Since the optimum osmolality for most living human cells is 280–300 mmol/kg H_2O, higher osmolalities cause shrinkage of cells with alteration of their density. 1·2M NaCl is therefore the recommended concentration.

2.2 INDIRECT DILUTION OF PERCOLL

Iso-osmotic Percoll with the optimum osmolality of 290–300 mmol/kg H_2O can be obtained by mixing 9 parts of the purchased Percoll with 1 part 1·2M NaCl (8%, w/v) and diluting to the desired density with balanced salt solution or Eagle's minimal essential medium. For the separation methods described here Percoll solutions with the desired density were prepared by the indirect method.

Materials

i. Percoll (Pharmacia Fine Chemicals): mix before use.
ii. 1·2M NaCl (8%, w/v) solution. Pass through a 0·45 μm Millipore filter for sterilization.
iii. Buffered salt solution (BSS).

Procedure

PREPARATION OF ISO-OSMOTIC PERCOLL

a. Mix 9 parts Percoll with 1 part of sterile 1·2M NaCl.
b. Measure the osmolality of the resulting iso-osmotic Percoll (280–300 mmol/kg H_2O).
c. Store in a sterile bottle at 4 °C.

DILUTION OF ISO-OSMOTIC PERCOLL

In the methods described in this chapter Percoll solution is required at four different densities, namely 1·077, 1·083, 1·09 and 1·11 g/ml. These can be prepared by adding BSS to the iso-osmotic Percoll in the proportions shown in *Table* 3.1.

Table 3.1. Preparation of Percoll solutions of different densities using the indirect dilution method

Percoll Density g/ml	*Equivalent volumes of solutions*	
	Iso-osmotic Percoll	*BSS or Eagle's MEM*
1·077	5	3
1·083	15	8
1·09	15	5
1·11	17	3

Comments

a. Using the indirect dilution method, enough gradient solution of the required density can be prepared in advance, thereby reducing the risk of variation of the density from experiment to experiment. In addition, less time is required for preparation of the wanted cells.
b. Iso-osmotic Percoll can also be diluted with Eagle's minimal essential medium (MEM).
c. For sterilization, pass through a 0·45 μm Millipore filter.

3. DETERMINATION OF PERCOLL DENSITY BY CALIBRATION CURVE

The simplest way to determine the density of a given Percoll solution is by measuring the refractive index of the solution with a refractometer. The refractive index has a linear correlation with density (Pertoft and Laurent, 1977).

Materials

i. Percoll (Pharmacia Fine Chemicals): record the density of the purchased Percoll which is always printed on the label.
ii. Buffered salt solution (BSS).

Equipment

Refractometer (Atago).

Procedure

PREPARATION OF PERCOLL SOLUTIONS

a. Prepare iso-osmotic Percoll as described in Section 2.2.

b. Make up a series of dilutions of iso-osmotic Percoll (20%, 50% and 80%) in BSS or Eagle's MEM.

ESTIMATION OF REFRACTIVE INDEX (RI).

a. Set the refractometer to the lowest point on the middle scale (1·333) using distilled water. Clean and dry the plate with tissue paper after each measurement. Place a small volume of BSS and record the RI.

b. Measure the RI of the purchased Percoll (RI = 1·3518).

c. On a graph of RI against density join the two points as shown in *Fig*. 3.1.

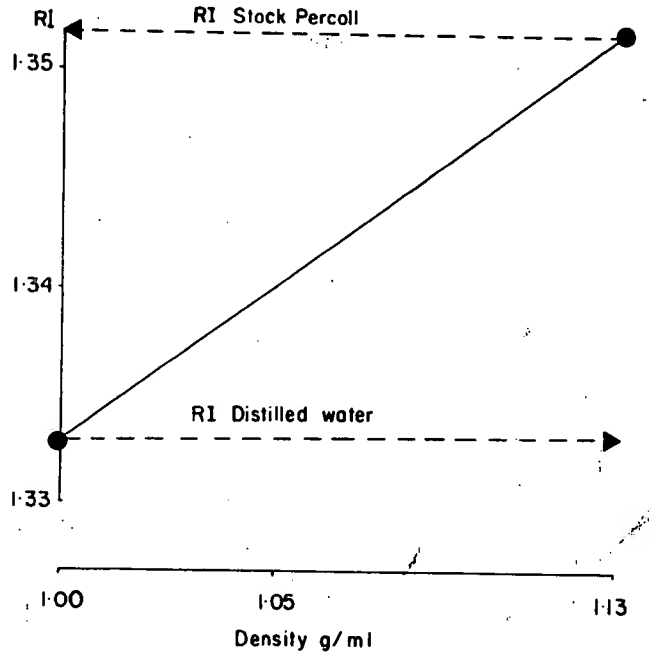

Fig. 3.1. The relationship between the density of purchased Percoll and its refractive index (RI).

d. Measure the RI of the iso-osmotic Percoll and then determine the RIs of the 20%, 50% and 80% soltions.

e. Plot another graph of RI against percentage iso-osmotic Percoll with best straight line drawn through the points as shown in *Fig*. 3.2.

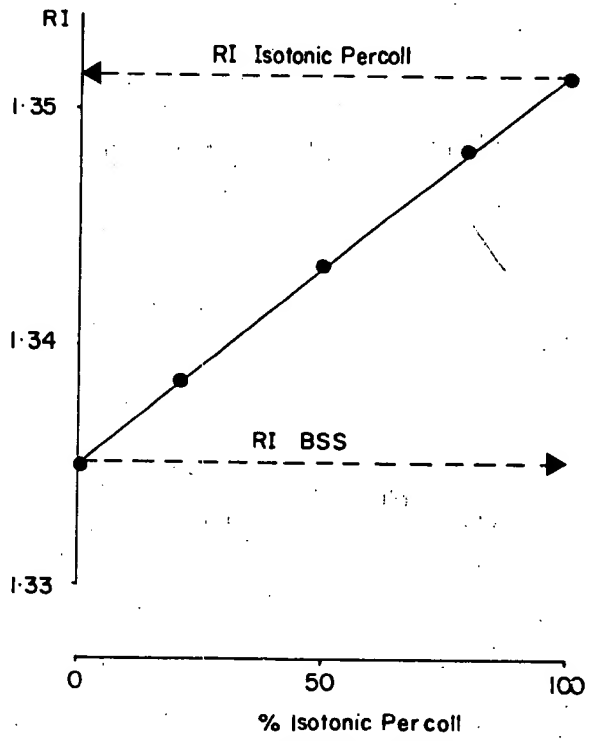

Fig. 3.2. The refractive indices (RI) of solutions containing different proportions of iso-osmotic Percoll and buffered salt solution (BSS).

APPLICATIONS

a. To determine the density of any Percoll solution, measure the RI and extrapolate.

b. The graph can also be used to prepare Percoll solution of any density. Use *Fig*. 3.1 to calculate the RI. From *Fig*. 3.2 determine the percentage dilution of iso-osmotic Percoll (with BSS or Eagle's MEM) to give the RI calculated from *Fig*. 3.1.

Comment

For good reproducibility a calibration curve should be plotted with each batch of the purchased Percoll.

4. SINGLE-STEP DENSITY GRADIENT CENTRIFUGATION OF CELLS (ISOPYKNIC CUSHIONING)

The procedure described here can separate a population of cells into

two layers. The first layer is formed at the interface between the suspending fluid and the Percoll solution, and the second layer is usually found as a sediment at the bottom of the tube (Ali et al, 1982).

4.1 HARVESTING OF MONONUCLEAR CELLS

Percoll of 1·077 g/ml density can be used for a rapid separation of blood mononuclear cell layer (MNL) (monocytes and lymphocytes) from the granulocytes and mature red cells.

Materials

i. Preservative-free heparin (Duncan Flockhart Co. Ltd): 15 units/ml blood.
ii. Percoll solution (density 1.077 g/ml): prepare as described in Section 2.2.
iii. Buffered salt solution (BSS).
iv. Heat-inactivated foetal calf serum (HI-FCS).
v. 0·45 μm filter (Millipore).

Procedure

a. Mix venous blood with heparin and prepare a buffy coat cell layer. Suspend the cells in the donor's own plasma (which has been centrifuged hard to sediment the platelets, *see* Comment 1) or in any physiological solution (BSS or Eagle's MEM).

b. Count the total number of nucleated cells, and prepare cytocentrifuge slides for Jenner–Giemsa staining.

c. Into a sterile container (or preferably a polycarbonate tube, supplied by MSE Scientific Instruments Ltd), carefully layer the buffy coat cell suspension onto an equal volume of Percoll solution using a syringe and 19 gauge needle.

d. Centrifuge at 400*g* for 15–20 min at room temperature in a bench centrifuge.

e. Discard the supernatant (plasma or suspending medium). This also removes some of the remaining platelets.

f. Harvest the mononuclear cells from the Percoll/plasma interface using a 19 gauge needle and syringe, and dispense 5 ml of cell suspension into each container. To recover all of the cells, also remove the medium lying immediately above the pellet of red cells and granulocytes.

g. To sediment the separated MNL, add BSS to a final volume of about 15–20 ml for each 5 ml cell suspension, to decrease the density of the Percoll solution.

h. Centrifuge at room temperature for 7–10 min at 1000*g*.

i. Suspend the pelleted cells in the donor's own plasma, HI-FCS or any suitable medium.

j. Mix well using a syringe and needle or a vortex mixer. Count the total number of cells and prepare Jenner–Giemsa stained cytocentrifuge slides for morphological examination and for obtaining a differential cell count.

k. Determine cell viability using trypan blue exclusion.

l. Estimate the recovery of MNL using the total number and the differential count at the beginning and end of the procedure as follows:

$$\% \text{ Recovery} = \frac{\text{Total MNL cell number collected finally}}{\text{Total MNL cell number initially in the blood}} \times 100$$

Reproducibility

Separation of normal blood cells by Percoll cushioning allowed harvesting of monocytes and lymphocytes at the Percoll/suspending medium interface, while the granulocytes and mature red cells were sedimented to the bottom of the tube. From seven consecutive experiments, the mean percentage of each cell type was: 82% (SE 3·0) lymphocytes; 17% (SE 3·0) monocytes and 0·85% (SE 0·3) granulocytes. Cell viability was high (more than 90%). The recovery of isolated cells was more than 80% and the morphological appearance of the cells appeared to be unchanged.

Comments

a. To use the donor's own plasma, centrifuge at 2350*g* for 7 min to sediment the platelets, and pass through a 0·45 μm Millipore filter.

b. For reasons of economy, it is necessary to use a buffy coat preparation rather than whole blood so that the volume of Percoll used can be kept to a minimum.

c. An alternative method for removing platelets from the MNL is by suspending the cells in a few drops of HI-FCS, and layer carefully onto 10 ml HI-FCS. Centrifuge at 400*g* for 15 min. Discard the supernatant HI-FCS and repeat the procedure (using 5 ml HI-FCS) before suspending the cells in the suspending fluid.

d. A comparative study was performed using both Percoll and Ficoll–Triosil (Pharmacia Fine Chemicals, Chapter 6, Section 1) for the isolation of peripheral blood mononuclear cells from both whole blood and buffy coat.

The data indicated that Percoll is as effective as Ficoll–Triosil in preparing MNL. However Percoll is the preferred medium since its lower viscosity (10 ± 5 cP at 20 °C) allows rapid separation of cells (15–20 min) when compared with Ficoll–Triosil (30–40 min).

4.2 PREPARATION OF PURE LYMPHOCYTES

Depletion of phagocytic cells from the mononuclear cell suspension to obtain pure lymphocytes can be achieved by incubation with carbonyl iron particles (Rothbarth et al, 1976). The carbonyl iron loaded monocytes can then be sedimented by centrifugation on a Percoll cushion of 1·077 g/ml density, due to their increased density (Ali et al, 1982).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.
ii. Carbonyl iron powder (Goodfellow Metals).
iii. Eagle's minimal essential medium (MEM).
iv. Buffered salt solution (BSS).
v. Heat-inactivated foetal calf serum; for cytocentrifuge slides.
vi. 0·45 μm filter (Millipore).

Equipment

Rotary mixer.

Procedure

STERILIZATION OF CARBONYL IRON POWDER

a. Place 1 g carbonyl iron powder into a clean, screw-capped bottle.
b. Sterilize by autoclaving at 15 lb/in^2 at 121 °C for 20 min.
c. Dry the bottle in an incubator at 37 °C. Store at room temperature.

INCUBATION WITH CARBONYL IRON

a. Mix whole blood with heparin and centrifuge (450 *g* for 15 min) to prepare the buffy coat. Place the buffy coat cell suspension and plasma in separate containers.
b. Centrifuge the plasma (2350 *g* for 7 min) to sediment the platelets and pass through a 0·45 μm Millipore filter. Suspend the cells in the filtered plasma (keep some of the plasma in a sterile container).
c. Prepare cytocentrifuge slides and stain with Jenner–Giemsa.
d. Prepare a mononuclear cell layer (MNL_1) using Percoll 1·077 g/ml as described in Section 4.1.
e. Count the total number of nucleated cells and prepare cytocentrifuge slides for both Jenner–Giemsa and non-specific esterase staining (Chapter 2).

f. Add 10 ml of Eagle's MEM to the sterile carbonyl iron powder, mix well and add 50 μl of this suspension to each 1 ml of the mononuclear cell suspension (contains about 3–5 × 10^6 nucleated cells).
g. Incubate the cell mixture for 45 min at 37 °C on a rotary mixer. Shake the tube occasionally using a vortex mixer (or by hand) to avoid sedimentation of the carbonyl iron particles and to prevent aggregation of the monocytes.

DEPLETION OF PHAGOCYTIC CELLS

a. With a syringe and 19 gauge needle resuspend the cell mixture and carefully layer onto an equal volume of 1·077 g/ml Percoll solution.
b. Prepare a second batch of mononuclear cell layer (MNL_2) and harvest them at the interface. Wash twice and suspend with BSS, Eagle's MEM or the patient's own plasma. The pellet will contain the carbonyl iron-loaded monocytes.
c. Count the total cell number and prepare stained cytocentrifuge slides for morphological examination and differential counting.
d. Calculate the recovery of lymphocytes at the interface from the total cell number at the first and second MNL as follows:

$$\% \text{ Recovery} = \frac{\text{Total lymphocyte number in } MNL_2}{\text{Total lymphocyte number in } MNL_1} \times 100$$

Reproducibility

Incubation of mononuclear cells with carbonyl iron depleted the cells of monocytes and pure lymphocyte preparations were obtained. The mean percentages of the remaining cells from four experiments were: lymphocytes 96% (SE 2·6); monocytes 1·8%; granulocytes 1·6%. The mean recovery of lymphocytes from three preparations was 95% (SE 1·5). The recovery of B-lymphocytes after carbonyl iron depletion of monocytes was measured on one occasion using direct fluorescence staining and was found to be complete. The morphology of the prepared lymphocytes appeared to be normal (*Fig.* 3.3).

Comment

EDTA anti-coagulant should be avoided because it chelates Ca^{2+} and Mg^{2+} which are necessary for the carbonyl iron phagocytosis by monocytes.

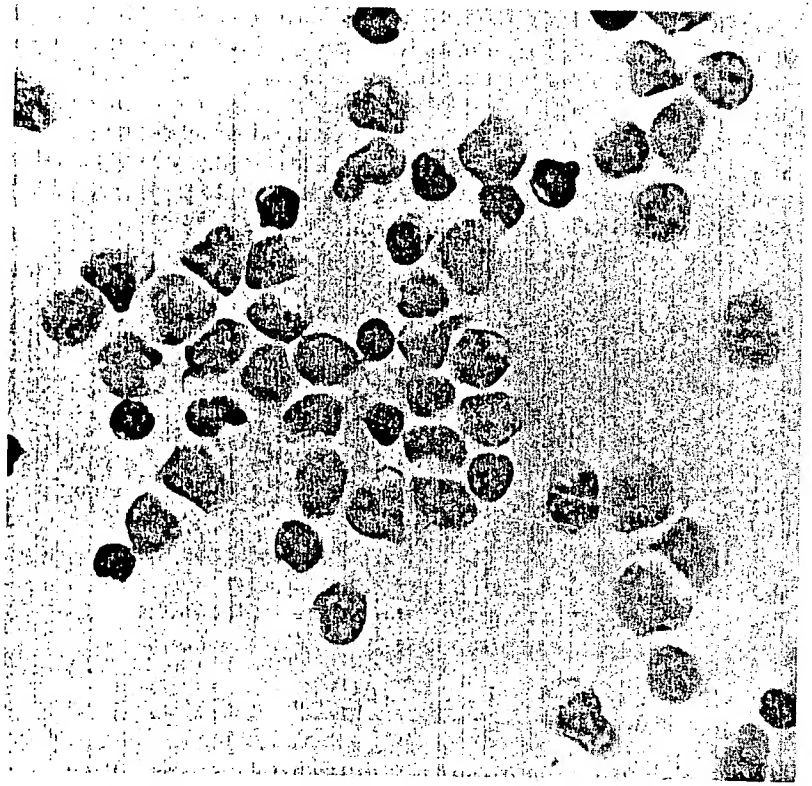

Fig. 3.3. Jenner–Giemsa staining of normal peripheral blood lymphocytes purified from mononuclear cells using carbonyl iron depletion of monocytes.

5. BANDING OF LEUCOCYTES BY DISCONTINUOUS PERCOLL GRADIENT

Banding of the mononuclear cell layer (MNL) on a discontinuous gradient of Percoll gives highly enriched monocyte and lymphocyte fractions. The methods described here can separate a population of cells into two bands, which form at the interface between Percoll layers of different densities during centrifugation. Cell separation by discontinuous Percoll gradients is useful when a high-speed centrifuge (which is required to generate the continuous density gradient of Percoll) is not available.

5.1 BANDING OF MONOCYTES AND LYMPHOCYTES

The method described here was established by Al-Sumidaie et al (1984).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.
ii. Eagle's MEM × 10 (Gibco): prepare iso-osmotic MEM by adding distilled water (1:9, v/v) and adjust to pH 7·4 with NaOH.
iii. Iso-osmotic Percoll solution: prepare as described in Section 2.2 using Eagle's MEM 10 ×.
iv. Percoll solution of 1·077 g/ml density (Section 2.2).
v. Buffered salt solution (BSS).
vi. Heat-inactivated foetal calf serum (HI-FCS).
vii. Polycarbonate tube (MSE Scientific Inst) *or* siliconized glass container (Appendix D).

Procedure

PERCOLL SOLUTIONS

Percoll solution is required at three different densities: 1·057, 1·066 and 1·074 (± 0·005) g/ml. These can be obtained by preparing 42%, 50% and 56·6% Percoll in iso-osmotic Eagle's MEM.

BANDING OF CELLS

a. Layer the heparinized blood or buffy coat cell suspension onto the same volume of Percoll solution (1·077 g/ml) (placed in a polycarbonate tube or a siliconized glass container) using a syringe and 19 gauge needle. Prepare MNL as described in Section 4.1. Wash the recovered cells twice and resuspend in cold BSS.

b. Count the total number of cells and prepare cytocentrifuge slides for staining with both Jenner–Giemsa and non-specific esterase (Chapter 2).

c. Centrifuge the MNL at 400 *g* for 7 min in a bench centrifuge.

d. Discard the supernatant and resuspend the pelleted cells with 2 ml of Percoll solution (1·074 g/ml).

e. Transfer the cells into a new tube and onto the surface of the cell suspension carefully layer using a syringe and needle 2 ml of Percoll solution (1·066 g/ml) by allowing the Percoll solution to run on the side wall of the tube.

f. Using the same technique, place the third layer of Percoll (1·057 g/ml) on the top of the two previous solutions (three layers should be visible).

g. Centrifuge the tube at 2200 *g* for 90 min at room temperature in a bench centrifuge.

h. Discard the supernatant Percoll solution (1·057 g/ml) above the mononuclear phagocyte (monocyte) band (*Fig.* 3.4) and collect the cell layer. Repeat the same technique to collect the next band of cells

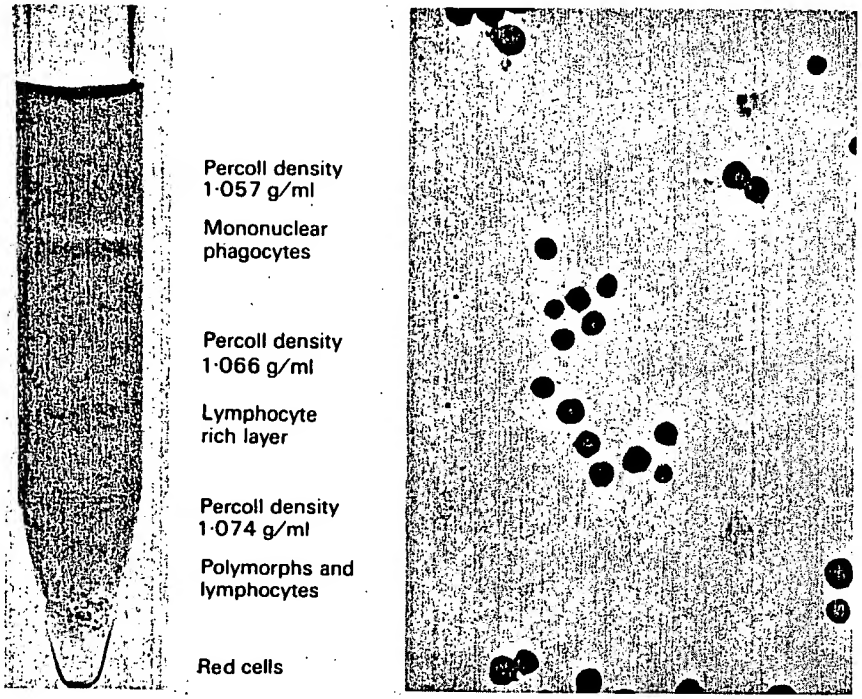

Fig. 3.4. Banding of leucocytes by a discontinuous density gradient of Percoll (left); non-specific esterase staining of monocytes (mononuclear phagocytes) obtained from the discontinuous density gradient (right).

(lymphocytes). The remaining cell band at the bottom of the tube contains polymorphs (neutrophils), some red cells and lymphocytes.

i. Count the total number of cells and prepare at least two cytocentrifuge slides for each band of cells. Stain with both Jenner–Giemsa and non-specific esterase (Chapter 2) to estimate the percentage of lymphocytes and monocytes in each cell band.

j. Check the viability of the recovered cells (if required) by dye exclusion of trypan blue.

k. Calculate the recovery of the nucleated cells (monocytes or lymphocytes) from the discontinuous gradient of Percoll as follows:

$$\% \text{ Recovery} = \frac{\text{Number of cells (monocytes or lymphocytes) recovered from all interfaces}}{\text{Number of cells (monocytes or lymphocytes) in the MNL cells}} \times 100$$

Reproducibility

Banding of mononuclear cells by a discontinuous density gradient of

Percoll produced highly enriched bands of monocytes and lymphocytes which formed at the interface between the Percoll layers (*Fig.* 3.4). From 100 experiments using normal subjects, the mean percentage of monocytes collected at the 42%–50% interface was 83% (SD ± 10) and the mean percentage of lymphocytes collected at the 50–56·6% interface was 92% (SD ± 6). Other bands (one at the top and the other at the bottom of the gradient) were discarded. The mean recovery of monocytes was 78% (SD ± 10) and of lymphocytes was 80% (SD ± 6). The morphology of the recovered cells was unchanged. The mean viability of monocytes was 97% and of lymphocytes was 98%.

Comments

a. It is preferable to use polycarbonate tubes to prevent adherence of monocytes with subsequent reduction in recovery.

b. Sufficient amounts of sterile Percoll of each density may be prepared in advance and stored at 4 °C. Solutions are stable at physiological pH and osmolality.

5.2 A RAPID METHOD FOR THE FRACTIONATION OF MONONUCLEAR CELLS

Mononuclear cells can also be fractionated into monocytes and lymphocytes by discontinuous density gradients of Percoll using a method reported by Weetman et al. (1983).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.
ii. Buffered salt solution (BSS).
iii. Iso-osmotic Percoll solution (Section 2).
iv. Heat-inactivated foetal calf serum (HI-FCS): for cytocentrifuge slide preparation.
v. Conical tube: 15 ml capacity.

Procedure

PERCOLL SOLUTIONS

Prepare 40%, 48% and 53% iso-osmotic Percoll in BSS.

FRACTIONATION OF CELLS

a. Collect venous blood in heprain and prepare a mononuclear cell layer (MNL) as described in Section 4.1. Wash the cells twice with BSS.

b. Suspend the pelleted mononuclear cells in 3 ml 53% Percoll solution in a conical tube and onto the top of this solution layer 3 ml 48% and 1 ml 40% Percoll solution using a syringe and needle (*see also* Section 5.1).

c. Centrifuge at 400 *g* for 20 min at room temperature.

d. Collect the two bands of cells and proceed as described in Section 5.1 (Step *h* onwards).

Reproducibility

Fractionation of MNL on a discontinuous density gradient of Percoll produced a significant enrichment of monocytes and lymphocytes in the fractions. In 14 experiments, more than 90% pure monocytes were collected at the 40–48% interface and more than 95% pure lymphocytes recovered from the bottom of the tube. Other bands were discarded. Purity was determined by non-specific esterase staining (Chapter 2). The morphology of the cells was unchanged. Recovery of the cells was variable (60–80%).

Comments

a. The monocyte fractions were irradiated with 3000 rads following isolation to eliminate contaminating lymphocytes but leaving monocyte function intact.

b. The separated cells were used to demonstrate production of thyroglobulin antibodies with autologous thyroglobulin-primed monocytes.

6. PREPARATION OF A CONTINUOUS DENSITY GRADIENT OF PERCOLL

A preformed continuous density gradient is stable and can be prepared up to a week in advance.

6.1 GENERATION OF THE GRADIENT

When a solution of Percoll in 0·12M NaCl is centrifuged at $< 10\,000\,g$ in an angle-head rotor, the coated silica particles will sediment in an uneven distribution thus forming a density gradient. Since Percoll is a polydispersed or heterogeneous colloid, its component particles sediment at different rates, producing a very smooth gradient. The gradient forms isometrically around the starting density and becomes progressively steeper with time. A gradient of Percoll formed by centrifugation will change continuously during high speed centrifugation. Prolonged centrifugation of Percoll at high *g* force results in all of the colloid sedimenting in a hard pellet at the base of the tube. Gradients of many shapes and ranges can be formed by varying the starting density and the centrifugation conditions. The 'S' shaped gradient curve is characterized by a fairly flat region occupying most of the centrifuge tube. This enables cells of very similar buoyant densities to be separated with high resolution.

The method described here can be used for the generation of 10, 20 and 50 ml Percoll gradients.

Materials

Percoll solution, of known starting density (Section 2.2).

Equipment

i. MSE 25 angle-head rotor (MSE Scientific Inst.).

ii. Polycarbonate tubes, with aluminium screw-top caps (MSE Scientific Inst.).

Procedure

a. Place the diluted Percoll solution into a polycarbonate tube and cap tightly. Prepare two gradients if density marker beads are used to monitor the gradients (*see* Section 6.2).

b. Centrifuge in an MSE 25 angle-head rotor, with the brake off, for 45 min at 20 000 *g* and 4 °C. Use a balanced polycarbonate tube containing either identical gradient materials or distilled water.

c. Allow the gradient to warm up to room temperature before use.

d. To store the gradient, keep the tube in a stable, upright position at 4 °C. Use preferably within 3 days.

Comments

a. A minimum of centrifugal force of about 10 000 *g* should be used for Percoll in 0·12M NaCl in order to self-generate gradients in angle-head rotors. Rotor geometry and tube size have a marked effect on the shape of the curve. As the angle of the rotor comes closer to the vertical, the path length for the formation of the gradient becomes shorter and the gradient forms more rapidly.

b. It is not possible to use swing-out rotors for self-generating gradients, due to the long path length and unequal *g* force along the tube.

6.2 DETERMINATION OF FRACTION DENSITY BY DENSITY MARKER BEADS

Density marker beads provide a simple and rapid method for measuring the fraction density of a continuous density gradient of Percoll. The position of the cells within the gradients can also be located. Therefore, density marker beads can be used as an external marker (for monitoring gradient shape and range) in a centrifuge tube containing identical gradient material to the one used for the experiment.

Materials

i. Percoll solution (10 ml) of a known density (Section 2.2): generate the density gradient as described in Section 6.1.
ii. Density marker beads (Pharmacia Fine Chemicals).
iii. Heat-inactivated foetal calf serum (HI-FCS).
iv. Polycarbonate tube (MSE Scientific Inst.): 15 ml capacity.

Procedure

PREPARATION OF BEAD SUSPENSION

Add 1 ml distilled water to each of the ten vials and leave the beads to swell for at least 18 h at 4 °C. Store at 4 °C and use when required.

CENTRIFUGATION ON PERCOLL GRADIENT

a. Transfer 20 µl (220 µl for 50 ml gradient) of bead suspension from the first nine vials (as suggested by the manufacturers) to a tube containing 1 ml (5 ml for 50 ml gradient) HI-FCS (use a new disposable tip for each vial). Mix well using a syringe and needle.

b. Gently, layer the bead suspension onto the preformed Percoll gradient and centrifuge at 1400 *g* for 15 min. This tube can be used as a counter-balance in the rotor during centrifugation of the tube containing the cell suspension.

c. Record the distribution of the coloured beads (*see Fig.* 3.5) throughout the gradient.

d. Measure the distance (in mm) of each band from the bottom of the tube using millimetre graph paper.

e. Plot a graph of density versus distance (mm) from the bottom of the tube (*Fig.* 3.6).

Comments

a. A simple but laborious method of measuring the density of the fractions without the use of a refractometer or density marker beads is to weigh a known volume of Percoll solution from each fraction.

b. In order to store the density marker beads, add merthiolate (0·01%, w/v) and do not freeze.

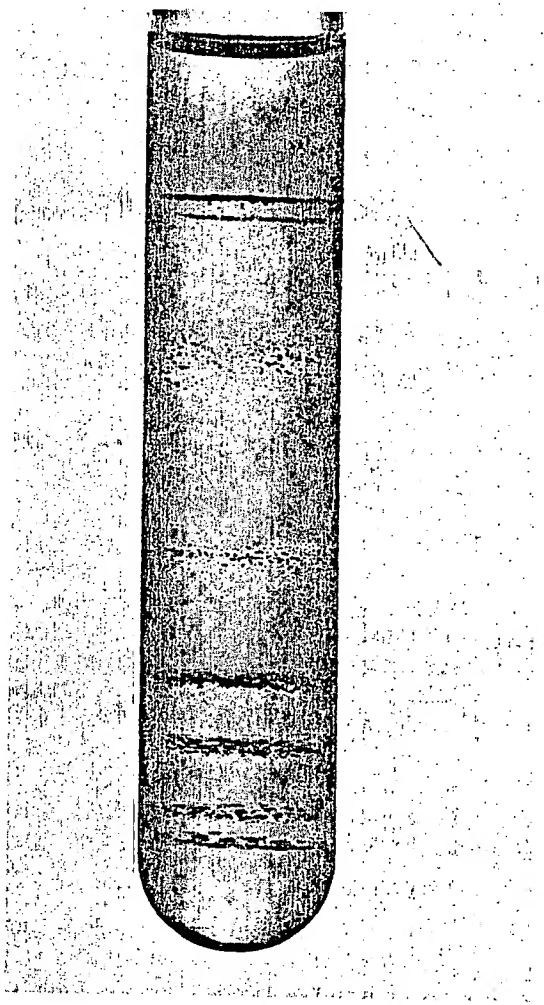

Fig. 3.5. Banding of density marker beads through a preformed Percoll gradient with starting density of 1·083 g/ml.

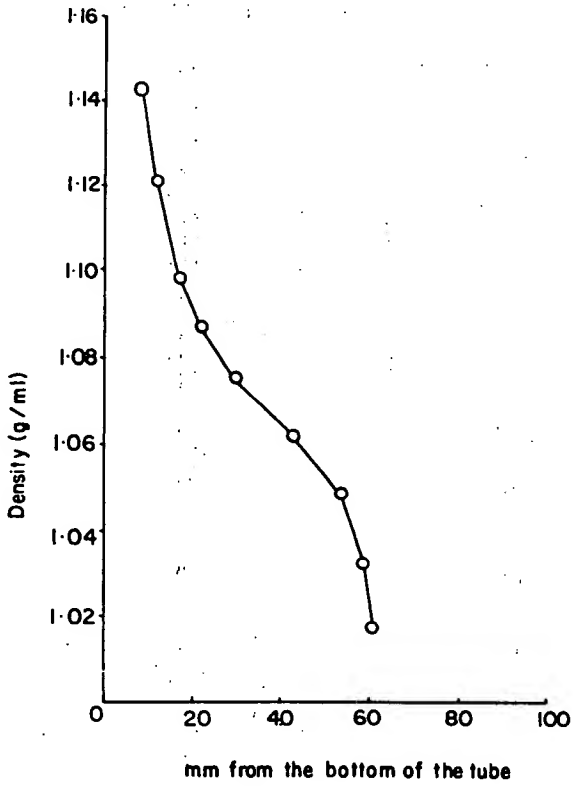

Fig. 3.6. The use of coloured density marker beads in the calibration of a preformed Percoll gradient of starting density of 1·083 g/ml.

7. FRACTIONATION OF LEUCOCYTES ON A PREFORMED PERCOLL GRADIENT

This method is useful for obtaining cell populations for which there is only a small difference in density between each cell type. Successful cell separation can be obtained with initial samples not exceeding 80×10^6 cells/ml for each of 10 ml of gradient materials.

7.1 FRACTIONATION OF MONONUCLEAR CELLS

Isopyknic centrifugation of mononuclear cells on a continuous density gradient formed from Percoll of starting density 1·083 g/ml can be used to separate monocytes from lymphocytes. The monocytes are greatly enriched in the low density fractions whereas fractions of higher density contain only lymphocytes. If neutrophils and eosinophils are present in the pre-Percoll layer of cells they can be located in the lower fractions of the gradient just above the red cell layer.

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.

ii. Percoll solution (1·077 g/ml): prepare as described in Section 2.2.

iii. Preformed density gradient of Percoll with starting density of 1·083 g/ml (Section 2.2): generate the gradient as described in Section 6.1.

iv. Buffered salt solution (BSS).

v. Heat-inactivated foetal calf serum (HI-FCS).

Procedure

CENTRIFUGATION OF CELLS

a. Layer the heparinized blood or buffy coat cell suspension onto an equal volume of Percoll (density 1·077 g/ml) and prepare a mononuclear cell layer (MNL) as described in Section 4.1. Suspend the recovered cells in 1 ml BSS or HI-FCS.

b. Count the total number of nucleated cells and prepare cytocentrifuge slides for Jenner–Giemsa staining.

c. Gently layer the cell suspension onto the top of the preformed gradient of Percoll, using a syringe and 19 gauge needle.

d. Centrifuge at 1400 *g* for 15 min in a bench centrifuge at room temperature.

FRACTIONATION OF GRADIENT MATERIAL

a. Discard 2 ml from the top of the gradient, and collect 0·5 ml fractions into a set of tubes using a 1 ml syringe and needle. Wash the syringe thoroughly with BSS after collection of each fraction.

b. To sediment the isolated cells, add 5 ml BSS to each tube, and centrifuge at 800 *g* for 7 min.

c. Wash the pelleted cells once in 2 ml BSS. Discard the supernatant and resuspend in 0·3–0·5 ml HI-FCS or BSS.

d. Count the total number of cells in each fraction, prepare cytocentrifuge slides and stain with Jenner–Giemsa for morphological evaluation and differential counting.

e. Check the viability by trypan blue exclusion.

Table 3.2 Fractionation of peripheral blood mononuclear cells using equilibrium density gradient centrifugation on Percoll of starting density 1·083 g/ml

Fraction No.	*Monocytes (%) Expt.*		*Lymphocytes (%) Expt.*		*Neutrophils (%) Expt.*	
	1	*2*	*1*	*2*	*1*	*2*
Pre-Percoll	16	15	76	84	7	0·5
3	—	84	—	16	—	0
4	90	77	9	23	1	0
5	45	56	54	44	1	0
6	19	31	80	69	0·6	0
7	0·4	5	97	92	2	3
8	0	11	97	85	3	4
9	0·2	2	90	96	9	2
10	0	2	49	87	51	11
11	0	1	18	89	82	10

f. Calculate the recovery of cells from the gradient as follows:

$$\% \text{ Recovery} = \frac{\text{Total cell number (monocytes or lymphocytes) collected from the fractions}}{\text{Total cell number (monocytes or lymphocytes) layered onto the gradient}} \times 100$$

Reproducibility

Continuous equilibrium density gradient centrifugation of mononuclear cells (prepared from normal blood samples) separated the monocytes from the bulk of the lymphocytes. In two experiments (*Table* 3.2) the monocytes were greatly enriched (90% and 84%) in the fractions of low density compared with the original mononuclear cells (16% and 15% in experiments 1 and 2). Certain fractions of higher density contained up to 97% lymphocytes. Neutrophils (if present) were found in the lowermost dense fractions of the gradient. For example, in the first preparation the fraction containing 82% neutrophils was enriched from 7% neutrophils originally in the mononuclear cell layer. Platelets contaminate the least dense fractions and bind to the monocytes. This was avoided to a significant extent by prior preparation of a buffy coat (*see* Chapter 2, Section 4). The morphology of the separated cells was found to be unchanged.

Comments

a. Cell clumps should be removed from the suspension before layering onto the Percoll gradient. This can be achieved by passing the cells through a sterile nylon gauze filter.

b. Sufficient amounts of the sterile gradient material can be prepared in advance and stored at 4 °C. This solution is stable at physiological pH and osmolality.

c. The above gradient (1·083 g/ml density) can be used for the separation of granulocytes from red cells. Centrifuge the heparinized blood on a Percoll cushion (1·077 g/ml density) as described in Section 2.2. Collect the layer of granulocytes just above the red cells at the bottom of the tube. Wash and layer the cells onto the preformed gradient. Collect cell fractions to just above the pink band of cells. Wash twice with BSS and prepare cytocentrifuge slides for Jenner–Giemsa staining to estimate the percentage of neutrophils.

7.2 SEPARATION OF B- AND T-LYMPHOCYTES

Equilibrium centrifugation of either blood mononuclear cells or of pure lymphocytes obtained by carbonyl iron (or glass bead adherence) removal of monocytes on a continuous density gradient of Percoll can be successfully used to isolate lymphocyte subpopulations. B-lymphocytes with surface immunoglobulin can be detected in the regions of low density and T-lymphocytes in the regions of higher density (as shown by sheep red cell rosetting). T_M-lymphocytes with their characteristic positive 'dot' pattern after non-specific esterase staining can be found mainly in the region of higher density. By equilibrium density gradient centrifugation, there is always a preferential enrichment of T-lymphocytes, either in the presence or absence of monocytes (Ali et al, 1982).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.
ii. Percoll solution 1·077 g/ml density (Section 2.2).
iii. Percoll solution of 1·083 g/ml density (Section 2.2): prepare the continuous density gradient as described in Section 6.1.
iv. Buffered salt solution (BSS).
v. Heat-inactivated foetal calf serum (HI-FCS): for cytocentrifuge slides.
vi. Carbonyl iron powder (Goodfellow Metals).
vii. 0·45 μm filter (Millipore).

Equipment

Rotary mixer.

Procedure

PREPARATION OF CELLS

a. Collect venous blood from a normal donor and place into a collecting vessel containing heparin. Prepare mononuclear cells using Percoll (Section 4.1).

b. To obtain autologous plasma, transfer about 10 ml of the blood into another tube, centrifuge at 1400 *g* for 7 min in a bench centrifuge. Collect and recentrifuge the plasma for 10 min at 2350 *g* to sediment the platelets. Sterilize by passing through a 0·45 μm Millipore filter.

c. Count the total number of nucleated cells in the remaining blood sample. Prepare cytocentrifuge slides for both Jenner–Giemsa and non-specific esterase staining (Chapter 2).

d. Retain a small volume of the cell suspension (about 0·3 ml containing 3×10^6 cells/ml) to estimate the percentage of T-lymphocytes.

e. Remove the monocytes either by incubation with carbonyl iron as described in Section 4.2, or by adherence, *see* Chapter 6.

FRACTIONATION OF CELLS

After preparation of pure lymphocytes, mix the cells using a needle and syringe and layer gently onto the preformed density gradient of Percoll. Centrifuge and collect the fractions as described in Section 7.1.

IDENTIFICATION OF ISOLATED CELLS

Use some of the recovered cells for surface immunoglobulin fluorescence staining (to detect B-lymphocytes) and for E-rosette formation (to detect T-lymphocytes). Determine the percentage of monocytes and T_M-lymphocytes in the fractions, for example by staining for non-specific esterase (*see* Chapter 2).

Reproducibility

T- AND B-LYMPHOCYTES

Equilibrium centrifugation of either blood mononuclear cells or pure lymphocytes on a continuous density gradient gave lymphocyte fractions containing between 92% and 99% T-lymphocytes as shown by sheep red blood cell rosetting. Simultaneous sedimentation of carbonyl iron-loaded monocytes gave a clear picture of the distribution of B-lymphocytes (with surface immunoglobulin) throughout the density gradient. *Table* 3.3 shows the results from the two experiments done in this way. Fractions containing up to 40% B-lymphocytes could

Table 3.3. Fractionation of monocyte-depleted mononuclear cells to study the distribution of B- and T-lymphocytes using equilibrium density gradient centrifugation on Percoll of starting density 1·083 g/ml

Fraction No.	**Density marker beads collected* Expt. No. 1	2	*10^6 × cells/ml* Expt. No. 1	2	*Lymphocytes (%)* Expt. No. 1	2	*Monocytes (%)* Expt. No. 1	2	*SIg^+ (%)* Expt. No. 1	2	*E-rosettes (%)* Expt. No. 1	2
Pre-Percoll	—	—	·	·	85	92	15	5	—	5	79	71
1	—	Blue	2·7	0·6	—	—	—	—	30	—	—	—
2	—	Blue and orange	1·7	1·3	—	96	—	4	39	—	—	26
3	—	Green	0·7	1·2	87	77	13	22	39	35	50	40
4	—	Green and few red	1·1	1·6	99	68	1	32	22	22	75	19
5	—	Red	4·5	1·6	100	66	0	33	5	19	80	45
6	—	Red	8·4	1·4	100	93	0	7	2	10	90	74
7	—	Few red	6·4	4·8	99	97	0·5	3	7	3	82	83
8	—	Nil	2·5	11·5	100	99	0	1	2	3	92	91
9	—	Blue	2·0	16·5	97	99	0	0·5	5	3	81	92
10	—	Blue	2·0	7·9	98	98	0	—	6	4	81	93

*The buoyant densities (g/ml) of density marker beads are: blue, 1·018; orange, 1·033; green, 1·049; red, 1·062; blue, 1·075; orange, 1·087.

be obtained. These fractions were contaminated with T-lymphocytes, null lymphocytes, residual monocytes and dead cells. Fractions enriched in T-lymphocytes (90% and 93%) were found in the pure lymphocyte fractions in the lower part of the gradient. Significant numbers of B-lymphocytes are still present in the more dense fractions containing a greater number of cells but in these fractions they are greatly out-numbered by the T-lymphocytes. A tube with the density marker beads was run in parallel with the test tube in experiment 2 and the densities of the B- and T-lymphocytes recovered were estimated. B-lymphocytes with surface immunoglobulin were found to be concentrated in the region of low density (1·03–1·065) g/ml and T-lymphocytes in the region of higher density (1·06–1·08) g/ml. From eight experiments, two using mononuclear cells, two using glass bead adherence removal of monocytes and four using carbonyl iron, the mean percentages of T-lymphocytes were found to be increased from 82% (range 73–88%, SE 3·0) in the pre-Percoll layer, to 95% (range 92–99%, SE 0·93) in the most T-lymphocyte-enriched fraction from the gradient, which was often the fraction containing most cells (*Table* 3.4). The mean percentage of the non-lymphoid cells (monocytes and granulocytes) in these gradient fractions was 2·5% (range 0·5–13%, SE 0·46). There was always more than one fraction that was highly enriched in T-lymphocytes. The recovery of

Table 3.4. Non-specific esterase staining of lymphocyte fractions obtained by continuous density gradient centrifugation on Percoll of 1·083 g/ml density (By courtesy of Elsevier Biomedical Press B.V.)

Fraction No.	*Lymphocytes (%)*	*T-lymphocytes (%)*	*T_M-lymphocytes (%)*	*Diffuse-staining lymphocytes (%)*
Expt. I.				
4	55	—	—	—
5	93	81	36	53
6	99	82	48	52
7*	100	93	68	33
8	100	91	66	35
9	97	74	76	21
10	83	—	51	32
Expt. II				
5	89	—	29	59
6	99	—	54	45
7	96	—	73	23
8*	100	—	81	19
9	100	—	83	17
10	90	—	80	10

*Fraction with the highest cell number.

T-lymphocytes from the gradient was measured in two experiments and was found to be 69% and 70%. On one occasion the recovery of B-lymphocytes from the gradient was measured and found to be 63%. The mean viability of the mononuclear cells remained the same (99% and 98%) before and after carbonyl iron depletion of the monocytes, but the value dropped from 98% to 94% after E-rosette formation. The mean viability of eight T-lymphocyte fractions obtained from the gradient, as measured by trypan blue exclusion before E-rosetting, was 96·9% (SE 0·63).

T_M-LYMPHOCYTES

In two experiments, the fractions were studied for non-specific esterase staining (*Table* 3.4). T_M-lymphocytes were found preferentially in the lower part of the gradient, and those with negative or diffuse staining in the upper part. Diffuse-staining lymphocytes included T_G-, B- and null lymphocytes but not all of those in the upper part of the gradient can be accounted for by the B- and null lymphocytes, which suggests that even though there was considerable overlap, T_G- and T_M-lymphocytes were separated to some extent on the basis of density, so that the major T-lymphocyte-containing fraction is also enriched in T_M-cells.

Comments

a. There was some variation in the distribution of T-lymphocytes on the gradient from the different experiments. This may be due to technical inconsistencies. These include variation in the volume of the cell suspension layered or collected from the gradient and, possibly, the application of *g* force by inadvertently using different places in the centrifuge rotor (i.e. different radii). Standardization of the running conditions should decrease the differences. In addition, differences in the degree of enrichment of T-lymphocytes in the fractions may be more apparent than real because of differences in the viability of lymphocytes affecting rosetting efficiency (Platsoucas and Catsimpoolas, 1980a). They may, however, simply reflect the use of different donors. In some cases the carbonyl iron-loaded monocytes that did not sediment to the bottom of the tube still contaminated the T-lymphocyte fraction. The number of carbonyl iron particles ingested by these monocytes was perhaps not sufficient for the increase in density required for their sedimentation to the bottom of the gradient. Therefore, it may be better to sediment the carbonyl iron-loaded monocytes first, using Percoll cushion (1·077 g/ml density) as described in Section 4.2, and then layer the remaining cells onto the preformed gradient.

b. T-lymphocytes are commonly prepared by E-rosetting with separation of the rosetted cells from non-rosetted by pelleting the former through 1·077 g/ml Ficoll or Percoll (Jondal et al, 1972; Feucht et al, 1980). The removal of sheep red blood cells and the rosetting procedure itself may involve some loss of viability (Kay et al, 1977). The binding of sheep red blood cells to T-lymphocytes may bring about metabolic changes which might not be wanted (Larsson et al, 1978; Bevan et al, 1980). Equilibrium density gradient centrifugation of human peripheral blood mononuclear cells is rapid and non-disruptive and other workers have shown that T-lymphocytes are unaffected by their contact with Percoll. It is concluded that this would be an extremely useful method for obtaining viable and highly enriched T-lymphocyte fractions from normal human peripheral blood, although their selective enrichment in T_M-cells should be remembered.

c. In two experiments, a small volume of the cell suspension in the fractions was used to estimate the percentage of cells that form EAC-rosettes (Chapter 2, Section 9.2). The results showed that between 10 and 20% EAC-rosettes can be obtained in the lower fractions (fraction 10 onwards). In one experiment, the rosetted cells were successfully sedimented by using Ficoll–Triosil and the attached SRBC were then removed by lysing with NH_4Cl (Chapter 6, Sections 1.1 and 7.4 respectively).

8. ENRICHMENT OF PERIPHERAL BLOOD RETICULOCYTES

Preformed density gradient of Percoll with starting density of 1·11 g/ml can be used for the fractionation of normal blood erythrocytes giving, in the most enriched fractions, a seven-fold enrichment of reticulocytes. The reticulocyte-rich fractions can be obtained relatively free of white cells (Peters, 1982).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.
ii. Percoll solution of 1·11 g/ml density (Section 2.2): generate the density gradient as described in Section 6.1.
iii. Buffered salt solution (BSS).
iv. Heat-inactivated foetal calf serum (HI-FCS).

Procedure

a. Place blood from a normal subject into a tube containing heparin and prepare a buffy coat.

b. Collect the buffy coat together with the top layer of red cells and plasma in separate containers.

c. Suspend the cells in 1 ml autologous plasma (which was centrifuged at a high speed to sediment the platelets) and mix well.

d. Carefully, layer the cell suspension onto the preformed gradient of Percoll.

e. Centrifuge at 1200 *g* for 15 min at room temperature.

f. Discard 2 ml supernatant from the top of the gradient together with the upper layer of cells until the first pink band of cells.

g. Collect at least 5 fractions (0·5 ml each) into separate tubes.

h. Wash the recovered cells twice with BSS.

i. Resuspend with 0·5 ml autologous plasma. Use a small volume of the cell suspension in each tube for reticulocyte staining (Chapter 2, Section 8.1) and estimate the percentage of reticulocytes in the suspension.

Reproducibility

Fractionation of human erythrocytes on a continuous density gradient of Percoll produced a significant enrichment in the percentage of reticulocytes. In two separate experiments, the top cell fractions (pink colorations) were still contaminated with white cells and were therefore discarded. However, in the first fraction collected with insignificant white cell contamination there was an enrichment of reticulocytes from 1·2% and 0·8% in the original buffy coat–red cell layer to 6·4% and 5·3% respectively. Lower percentages of reticulocytes were obtained further down the gradient.

Comments

a. The seven-fold enrichment of reticulocytes from normal blood was sufficient for the study of globin chain synthesis in normal blood (Peters et al, 1983a). These studies showed that reticulocyte fractionation on Percoll made no difference to the results obtained and therefore leaves these cells metabolically intact.

b. The percentage of reticulocytes in the reticulocyte-rich layer was only about 7%, but this could be improved by taking a smaller (0·2 ml) fraction of cells.

Further applications

The above method has also been used for the following studies:

a. Equilibrium density centrifugation of a blood sample obtained from a patient with non-microspherocytic haemolytic anaemia increased the percentage of reticulocytes from 24% in the original red

cell layer (pre-Percoll layer) to 98% and 95% (duplicate separation) in the top fractions. The lower fractions collected from both gradients contained less than 0·5% reticulocytes. Red cells of different stages of maturation were then used for the measurement of hexokinase activity (*see* Chapter 1, Section 1.2) (M. Wagstaff, Department of Haematology, Welsh National College of Medicine, Cardiff).

b. Since the density of red cells is dependent upon their intracellular haemoglobin concentration, and Percoll seems to have no detrimental effect on the metabolism of these cells, then equilibrium density centrifugation on Percoll provides a rapid means for separating two populations of red cells if they differ in terms of intracellular haemoglobin concentration, as for example occurs in some cases of sideroblastic anaemia (A. May and S. Peters, unpublished).

9. ISOLATION OF CORD BLOOD AND EARLY POST-NATAL BLOOD RETICULOCYTES

Preformed gradients of Percoll with increasing density (1·09 g/ml) can be used to prepare highly enriched reticulocyte fractions. Fractionation of cord blood and early post-natal blood on this gradient can yield fractions containing up to 95% and 30% reticulocytes respectively when starting with reticulocyte percentages of 2–20% and about 2% respectively in the original samples (Sweet, 1985).

Materials

i. Preservative-free heparin (Duncan Flockhart Co Ltd): 15 units/ml blood.

ii. Buffered salt solution (BSS).

iii. Percoll solution of density 1·09 g/ml (Section 2.2): generate the gradient as described in Section 6.1.

iv. Heat-inactivated foetal calf serum (HI-FCS).

Procedure

a. Layer the heparinized blood on top of the preformed Percoll gradient.

b. Centrifuge for 15 min at 1200 *g* at room temperature.

c. Remove the upper white cell bands to just above the pink cell layer, using a syringe and needle.

d. Collect the pink band of cells, which contains most of the reticulocytes, to just above the dark red cell layer.

e. Wash the collected cells twice with BSS.

f. Suspend the pelleted cells with HI-FCS and centrifuge at 400 *g* for 10 min to remove the BSS.

g. Resuspend in HI-FCS. Use a small volume of the cell suspension for brilliant cresyl blue staining to estimate the percentage of reticulocytes (Chapter 2, Section 8.1.).

Second Edition

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

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Contents

12 Physical Methods of Cell Separation

13 Characterization

14 Induction of Differentiation

15 The Transformed Phenotype

19 Measurement of Cytotoxicity and Viability

20 Culture of Specific Cell Types

21 Culture of Tumor Tissue

22 Three-Dimensional Culture Systems

23 Specialized Techniques

Analysis. Cell counting on beads can be difficult, so growth rate should be checked by determination of DNA (see Chapter 18), or protein if nonproteinaceous beads are used, or dehydrogenase activity using the MTT assay (see Chapter 19).

Variation. Most variations on the method arise from the choice of bead or design of the culture vessel and stirrer [Griffiths, 1986].

Many other mass culture techniques exist [McLimans, 1979] but they are of such specialized application that they will not be described in detail here. Linbro produced a multiplate system, similar to the Sterilin Chamber, but with plates at right angles to the long axis of the chamber. This resembled the multiplate system of Schleicher [1973] but was smaller. Amicon and Endotronics supply larger perfusion chambers in a similar style to the Vitafiber system (see above). The potential of these systems for large-scale high-density culture has yet to be explored, but they may be valuable in recreating high tissue-like cell densities both for production of natural substances and for synthesizing large numbers of cells in a tissue-like matrix.

Millipore has introduced a large-scale culture system (MCCS) for adherent cells based on a filter membrane as a support, which also allows for perfusion of the culture. As long as current restrictions on the use of transformed cells in biotechnology exist, there will be a need for mass culture systems for anchorage-dependent cells.

LYMPHOCYTE PREPARATION

There is a variety of methods for the preparation of lymphocytes, but flotation on a combination of Ficoll and sodium metrizoate (e.g., Hypaque) is still most widely used [Boyum, 1968a,b; Perper et al., 1968].

Outline

Whole citrated blood or plasma depleted in red cells by dextran accelerated sedimentation is layered on top of a dense layer of Ficoll and sodium metrizoate. After centrifugation most of the lymphocytes are found at the interface between the Ficoll/metrizoate and the plasma.

Materials

Blood sample
clear centrifuge tubes or universal containers
Dextraven 110 (Fisons)
PBSA
Lymphoprep (Flow) (Ficoll/metrizoate, adjusted to 1.077 g/cc (Pharmacia, Nygaard))
centrifuge
syringe or Pasteur pipette
serum-free medium
hemocytometer or cell counter

Protocol

1.
Add Dextraven 110 to blood sample to final concentration of 10% and incubate at 36.5°C for 30 min to allow most of the erythrocytes to sediment.
2.
Collect supernatant plasma, dilute 1:1 with PBSA and layer 9 ml onto 6 ml Lymphoprep or other Ficoll/sodium metrizoate mixture. This should be done in a wide transparent centrifuge tube with a cap such as the 25-ml Sterilin or Nunclon Universal Container, or the clear plastic Corning 50-ml tube, using double the above volumes.
3.
Centrifuge for 15 min at 400 *g* (measured at center of interface).
4.
Carefully remove plasma/PBSA without disturbing the interface.
5.
Collect the interface with a syringe or Pasteur pipette and dilute to 20 ml in serum-free medium (e.g., RPMI 1640 [Moore et al., 1967]).
6.
Centrifuge at 70 *g* for 10 min.
7.
Discard supernatant fluid and resuspend pellet in 2 ml serum-free medium. If several washes are required, e.g., to remove serum factors, resuspend cells in 20-ml serum-free medium, and centrifuge two or three times more, and finally resuspend pellet in 2 ml.
8.
Count cells on hemocytometer (count only nucleated cells) or on electronic counter.

Lymphocytes will be concentrated in the interface, along with some platelets and monocytes. Granulocytes will be found mostly in the Ficoll/metrizoate and in the 4 hr pellet, and erythrocytes will pellet at the bottom of the tube. Removal of monocytes and residual granulocytes can be achieved by their adherence to glass (beads or flask surface) or to nylon mesh. If purer preparations are required, fractionation on den-

sity gradients of metrizamide (Nygaard) or Percoll (Pharmacia) or by centrifugal elutriation (see Chapter 12) may be attempted. Alternatively, specific subpopulations of lymphocytes may be purified on antibody or lectin-bound affinity columns (Pharmacia).

Blast Transformation [Hume and Weidemann, 1980]

Lymphocytes in purified preparations, or in whole blood, may be stimulated with mitogens such as phytohemagglutinin (PHA), pokeweed mitogen (PWM), or antigen [Berger, 1979]. The resultant response may be used to quantify the immunocompetence of the cells. PHA stimulation is also used to produce mitosis for chromosomal analysis of peripheral blood [Kinlough and Robson, 1961; Rothvells and Siminovitch, 1958].

Materials

Medium + 10% FBS or autologous serum
phytohemaglutinin (PHA), 50 μg/ml
test tubes or universal containers
microscope slides
Colcemid, 0.01 μg/ml in BSS
0.075 M KCl

Protocol

1.
Using the washed interface fraction from step 7 above, incubate 2×10^6 cells/ml in medium, 1.5–2.0 cm deep, in HEPES or CO_2-buffered DMEM, CMRL 1066, or RPMI 1640 supplemented with 10% autologous serum or fetal bovine serum.
2.
Add PHA, 5 μg/ml (Final), to stimulate mitosis from 24 to 72 hr later.
3.
Collect samples at 24, 36, 48, 60, and 72 hr and prepare smears or cytocentrifuge slides to determine optimum incubation time (peak mitotic index).
4.
Add 0.001 μg/ml (final concentration) Colcemid for 2 hr when peak of mitosis is anticipated [Berger, 1979].
5.
Centrifuge cells after Colcemid treatment, resuspend in 0.075 M KCl for hypotonic swelling, and proceed as for chromosome preparation in Chapter 13.

AUTORADIOGRAPHY

The following description is intended to cover autoradiography of any small molecular precursor into a cold acid-insoluble macromolecule such as DNA, RNA, or protein. Other variations may be derived from this or found in the literature [Rogers, 1979; Stein and Yanishevsky, 1979].

Isotopes suitable for autoradiography are listed in Table 23.1. A low energy emitter, e.g., ^3H or ^{55}Fe, in combination with a thin emulsion, gives high intracellular resolution. Slightly higher energy emitters, e.g., ^{14}C and ^{35}S, give localization at the cellular level. Still higher energy isotopes, e.g., ^{131}I, ^{59}Fe, and ^{32}P, give poor resolution at the microscopic level but are used for autoradiographs of chromatograms and electropherograms where absorption of low energy emitters limits detection. Low concentrations of higher energy isotopes (^{14}C and above) used in conjunction with thick nuclear emulsions produce tracks useful in locating a few highly labeled particles, e.g., virus particles infecting a cell.

Tritium is used most frequently for autoradiography at the cellular level because the β-particles released have a mean range of about 1 μm, giving very good resolution. Tritium-labeled compounds are usually less expensive than the ^{14}C- or ^{35}S-labeled equivalents and have a long half-life. Because of the low energy of emission, however, it is important that the radiosensitive emulsion is positioned in close proximity to the specimen, with nothing between the cell and the emulsion. Even in this situation only the top 1 μm of the specimen will irradiate the emulsion.

β-particles entering the emulsion produce a latent image in the silver halide crystal lattice within the emulsion at the point where they stop and release their energy. The image may be visualized as metallic silver grains by treatment with an alkaline reducing agent (developer) with subsequent removal of the remaining unexposed silver halide by an acid fixer.

TABLE 23.1 Isotopes Suitable for Autoradiography

Isotope	Emission	Energy (mV) (mean)	T½
^3H	β^-	0.018	12·3 yr
^{55}Fe	X-rays	0.0065	2·6 yr
^{125}I	X-rays	0.035 0.033	60d
^{14}C	β^-	0.155	5568
^{35}S	β^-	0.167	87d
^{45}Ca	β^-	0.254	164d